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TITLE: Pharmacological Prevention and Reversion of Erectile Dysfunction after Radical Prostatectomy, By Modulation of Nitric Oxide/Cgmp Pathways

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During Year 1 we aimed to determine the time course of histological and functional changes affecting the penile corpora cavernosa after bilateral cavernosal nerve resection (BCNR) in the rat, as an experimental model for erectile dysfunction subsequent to radical prostatectomy for prostate cancer. This condition seriously affects the quality of life of male patients and their partners. We have now determined that this neuropraxia causes the loss of smooth muscle cells by apoptosis and then an excessive collagen deposition in the penile corpora cavernosa. This in turn is responsible for the impaired corporal compliance leading to corporal veno-occlusive dysfunction (CVOD), seen in these patients, that is in part counteracted by the spontaneous induction of inducible nitric oxide synthase (iNOS) that stimulate nitric oxide and cGMP production. Long-term continuous oral administration of PDE5 inhibitors immediately after BCNR, thus raising cGMP levels, prevents CVOD and the underlying histopathology of the corpora cavernosa. This may soon be translated into the clinic, once the appropriate dosing is established, as a treatment to prevent or counteract erectile dysfunction after radical prostatectomy.

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## Introduction

During Year 1 we aimed to determine the time course of histological and functional changes affecting the penile corpora cavernosa after bilateral cavernosal nerve resection (BCNR) in the rat, as an experimental model for erectile dysfunction subsequent to radical prostatectomy for prostate cancer. This condition seriously affects the quality of life of a large fraction of male patients undergoing this operation and their partners. Therefore, studying the mechanism that triggers it and trying to develop a pharmacological therapy aiming to cure this type of erectile dysfunction, have considerable public health significance.

Specifically in Aim 1, experiment 1 the objective was to determine whether the loss of smooth muscle cells (SMC) in the penile corpora cavernosa of the rat with BCNR occurs by apoptosis and precedes the intensification of collagen synthesis and the appearance of corporal veno-occlusive dysfunction (CVOD), or venous leakage, and what was the progression of spontaneous iNOS induction, an endogenous antifibrotic mechanism, during the 90 days time course.

In addition we aimed to advance in the experimental completion of this aim by partially addressing Experiment 2, namely whether PDE5 inhibitors given orally and continuously for a 45 day period could prevent the development of CVOD and the underlying histopathology

## Description of research accomplishments

### A. For Experiment 1

In our time table of work, we had projected that during Year 1 we would carry out Experiment 1 within Aim 1. According to the SOW, this experiment was described as follows:

**Exp 1. Time-course of BCNR induction of CVOD and the underlying penile SM fibrosis.** The aim is to determine the time course of CVOD and the underlying changes in collagen and SM content in the corpora cavernosa. The following groups of animals (n=8/group) will be used for testing erectile function and histological analysis of the penile corpora, after injection of a Col I-Pr-Bgal plasmid and BrdUr.

A-1-5) BCNR, sacrificed at 10, 14, 30, 60, or 90 days.

B-1-5) sham-operated, sacrificed at 10, 14, 30, 60, or 90 days.

In our project, we had predicted the following outcomes:

The histological alterations (fibrosis, loss of SMC) will be minimal in BCNR as compared to sham-operated animals at early periods, and only later on, at 60 and 90 days, they will become detectable, most likely more intense than what we observed at the previously reported 45 days. However, TGFβ1 may be expressed earlier. The CVOD at these late stages will affect the papaverine-ICP response because of this intensified corporal fibrosis

We have virtually finished with this experiment, although with the following variations: a) we introduced two early periods (1, 3 days) to verify the immediate changes occurring after BCNR. Because of this we had to omit the originally proposed 60 days period, but we do not consider this important since it is too close to the 45 days period as to yield significant information. In addition, the 10 days period was shifted to a 7 days period to make it more meaningful in terms of early changes.

Another change was to eliminate from the protocol the injection of BrdUr, because it is a mutagenic substance objected by our institutional IACUC and health safety committee, and can

be replaced by performing PCNA immunostaining on the tissue sections to detect replicating cells. The results are comparable between both procedures. We had communicated this to DOD before initiating this project.

Our initial results were communicated to the 2007 meeting of the American Urological Association (see abstract #1), and most of the data have been compiled on a manuscript submitted for publication (see paper #1). Both reports are enclosed in the Appendix, and we reproduce below the summary of the paper. The results, nine figures, and discussion are presented in full in this paper.

**Summary of paper #1 Fibrosis and loss of smooth muscle in the corpora cavernosa precede corporal veno-occlusive dysfunction (CVOD) induced by experimental cavernosal nerve damage in the rat.**

**Background and Objectives**. CVOD is present in the rat at 45 days after bilateral cavernosal nerve resection **(BCNR)**, a model for erectile dysfunction in men subsequent to radical prostatectomy. This is associated with a loss of smooth muscle cells **(SMC)** and fibrosis of the penile corpora cavernosa, accompanied by the expression of inducible nitric oxide synthase **(iNOS)** which is assumed to be vasculoprotective and antifibrotic. We have now studied the temporal relationship of these processes to determine whether CVOD occurs after corporal fibrosis and SMC loss and whether iNOS induction increases steadily throughout.

**Methods**. Fisher 344 male rats were subjected to either BCNR or sham operation, and then to cavernosometry after 1, 3, 7, 15, 30, and 45 days (n=8/group). Penile shaft tissue sections were subjected to Masson trichrome, immunodetection for alpha smooth muscle actin **(ASMA)** as SMC marker, iNOS, neuronal NOS (nNOS), endothelial NOS (eNOS), proliferating cell nuclear actin **(PCNA)** for cell proliferation, and TUNEL for apoptosis, followed by quantitative image analysis. Quantitative western blot measured some of these markers in tissue homogenates

**Results.** CVOD appeared late (30 days) in the BCNR rats as compared to the sham controls, and exacerbated at 45 days, and even more by L-NIL. In contrast, the SMC/collagen ratio in the BCNR corpora was reduced early (7 days) and bottomed at 30 and 45 days, due in part to the reduction of SMC, presumably caused by an increase in apoptosis peaking at 3 days but remaining high thereafter. Cell proliferation also peaked at 3 days but then normalized. nNOS was reduced early (3-7 days) and disappeared at 30 days, whereas eNOS was not affected. iNOS was induced at day 3, and steadily increased to high levels peaking at 30 days.

**Conclusions**. CVOD develops in the BCNR rat as a result of the early loss of corporal SMC by the neuropraxia-induced apoptosis, which the initial cell replication response cannot counteract, followed by fibrosis. The absence of eNOS decrease suggests that the endothelium is not considerably affected. The time course of iNOS induction and the previous demonstration of CVOD and fibrosis increase by the iNOS inhibitor L-NIL support an antifibrotic role for iNOS.

We are currently analyzing the penile tissue sections corresponding to the 90 days BCNR group that we intend to present in a separate paper, since there appears to be amelioration of CVOD that needs confirmation by repeating the cavernosometry. It is unexpected to find a spontaneous improvement of the vasculogenic erectile response, and therefore it is important to determine whether the reduction in the SMC/collagen ratio in the corpora cavernosa, and hence the impaired compliance, is partially corrected. We also intend to include the measurement of the erectile response to electrical field stimulation (EFS) of the cavernosal nerve, to determine whether there is some nerve repair that would restore cavernosal neurotransmission after the damage caused by the initial 0.4 mm nerve resection. Further proof will come from ongoing fluorogold injection experiments to determine by retrograde tracing whether some neural regeneration has occurred.

Another important ongoing determination is the measurement of the activation of the collagen I promoter in the corpora cavernosa at different periods after BCNR, that serves to indicate collagen synthesis. We present in the figure (additional to the over 20 figures on the attached papers) the results from the measurement by luminometry of β-galactosidase as a reporter protein in the penile homogenates, comparing with the sham control animals, at 14 and 90 days period.

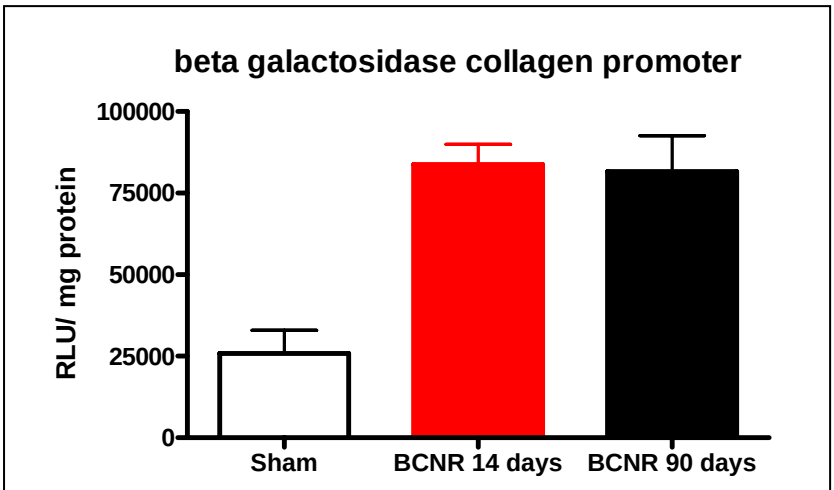


Figure 1: Activation of collagen synthesis by BCNR, determined by the use collagen promoter plasmid-link with beta galactosidase as a reporter gene; RLU : relative luminometry units

This shows that collagen synthesis in the corpora cavernosa remains at a late period (90 days) as high as at the intermediate period of 14 days, both compared to the levels found in sham-operated animals, thus suggesting that corporal fibrosis has not been corrected despite the apparent improvement of erectile dysfunction. Therefore, it is possible that other factors not necessarily related to smooth muscle loss or fibrosis are operating in the recovery of corporal SMC relaxation. They may be functional (e.g, higher nitric oxide or cGMP availability), or structural (phenotype changes in contractile proteins in the SMC?), and we will try to clarify them during Year 2, since it may well be that histological and functional regeneration of the corpora cavernosa in the rat is much faster and efficient than in men. Still, recent data discussed in paper 1 show that nerve sparing techniques during radical prostatectomy can save potency in nearly 2/3 of men one year after the surgery.

**B. For Experiment 4**

In our time table of work, we had projected that only during Year 3 we would carry out Experiment 4 within Aim 2. According to the SOW, this experiment was described as follows:

**Aim 2 Experiment 4. Time course of BCNR effects on collagen and SMC turnover rates, oxidative stress, and nitrosative reaction, and modulation of these processes by selected PDE5 inhibitor.** The aim is to investigate the effect of PDE5 inhibitors on the time course of alterations in collagen and SMC turnover and iNOS induction caused by BCNR.

Tissue sections and homogenates from all groups in Experiment 1 (time course, untreated) and from only one selected treatment paradigm in Experiments 2,3 will be utilized for assays.

Despite this experiment was not included in our program for Year 1, we have obtained some preliminary data. As explained and discussed above, we have carried out some measurements for the control untreated BCNR groups, such as PCNA/TUNEL for SMC, collagen I promoter activation for collagen synthesis, and iNOS/nNOS/eNOS expression. The results will be collectively presented when this experiment is finished in Year 2.

**C. For Experiment 2**

In our time table of work, we had projected that during Year 2 we would carry out Experiment 2 within Aim 1. According to the SOW, this experiment was described as follows:

**Aim 1. Experiment 2. Effects of continuous oral administration of PDE5 inhibitors or NO generators on CVOD and the underlying corporal fibrosis, at a selected time after BCNR.** The aim is to investigate whether the BCNR-induced corporal fibrosis can be prevented at longer periods by either long-term continuous treatment with PDE5 inhibitors or a NO generators.

Rat groups will be injected as in Exp 1 and treated for 90 days with:

1) Vardenafil, in the drinking water
2) Molsidomine, in the drinking water
3) Vardenafil and molsidomine given together in the drinking water

In our project, we had predicted the following outcomes:

Even if CVOD and fibrosis are more severe at 90 days, treatment with oral vardenafil will prevent these alterations, and similar effects will be achieved with molsidomine. The combination of both types of oral agents may be more efficacious, as proposed for PDE5 inhibitors bearing an NO donor in the same molecule

Despite this experiment was not included in our program for Year 1, we have advanced considerably by carrying the PDE5 inhibitor arm study. However, since vardenafil (Levitra) was already completed at the time of this award (Ferrini MG, Davila HH, Kovanecz I, Sanchez SP, Gonzalez-Cadavid NF, Rajfer J. Vardenafil prevents fibrosis and loss of corporal smooth muscle that occurs after bilateral cavernosal nerve resection in the rat. Urology. 2006 Aug;68(2):429-35), we decided to test also sildenafil (Viagra) and tadalafil (Cialis) in the UCNR (unilateral CNR) and BCNR models. Sildenafil has a short half life, similar to vardenafil, whereas tadalafil exerts more prolonged effects

The data are presented on two separate papers (see papers #2 and #3 in the Appendix). We reproduce below the summary of these papers. The results, figures, and discussion are presented in full in these papers.

**Summary of paper #2 Long-term continuous sildenafil treatment ameliorates corporal veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats**

It was recently reported in the rat that vardenafil given in a continuous long-term manner was successful in preventing smooth muscle fibrosis in the penile corpora cavernosa and corporal veno-occlusive dysfunction (CVOD) that occur following bilateral cavernosal nerve resection (BCNR), a model for human erectile dysfunction after radical prostatectomy. To expand on this finding and to determine whether this effect was common to other PDE5 inhibitors, and occurred in part by stimulation of the spontaneous induction of inducible nitric oxide synthase (iNOS, also known as NOS2), male Fischer 344 rats (N=10/group) were subjected to either BCNR or unilateral cavernosal nerve resection (UCNR) and treated with sildenafil (20 mg kg(-1) day(-1)) in the drinking water daily for 45 days. Additional BCNR groups received L-NIL (6.7 mg kg(-1) day(-1)) as inhibitor of iNOS activity, with or without concurrent sildenafil administration. It was determined that sildenafil, like vardenafil, (1) prevented the 30% decrease in the smooth muscle cell/collagen ratio, and the 3-4-fold increase in apoptosis and reduction in cell proliferation, and partially counteracted the increase in collagen, seen with both UCNR and BCNR; and (2) normalized the CVOD, measured by dynamic infusion

cavernosometry, induced by both BCNR and UCNR. The long-term inhibition of iNOS activity exacerbated corporal fibrosis and CVOD in the BCNR rats, but sildenafil functional effects were not affected by L-NIL. These data suggest that the salutary effects of continuous long-term PDE5 inhibitors on erectile function post-cavernosal nerve resection involve their ability to prevent the alterations in corporal histology induced by cavernosal nerve damage, in a process apparently independent from endogenous iNOS induction.

**Summary of paper #3 Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction that occurs after cavernosal nerve resection**

**OBJECTIVES:** To determine whether a long-term single daily oral dose of a longer half-life phosphodiesterase-5 (PDE5) inhibitor, tadalafil, has a similar effect to that of the shorter half-life PDE5 inhibitors sildenafil and vardenafil, and can prevent the fibrosis and resultant corporal veno-occlusive dysfunction (CVOD) occurring after cavernosal nerve (CN) injury.

**MATERIALS AND METHODS**: Male rats (10 per group) had either a sham operation, unilateral CN resection (CNR) or bilateral CNR, and were left untreated or given retrolingually 5 mg/kg per day of tadalafil. After 45 days, CVOD was assessed via cavernosometry, and the underlying corporal tissue changes were examined by immunohistochemistry and histochemistry (followed by quantitative image analysis), Western blots, and ad hoc methods.

**RESULTS**: Tadalafil treatment normalized the low response to papaverine and high drop rate in the intracavernosal pressure measured by cavernosometry after CNR compared with sham-operated rats. Tadalafil also normalized the increase in penile shaft collagen content, and the reduction in corporal smooth muscle cell (SMC) content, SMC/collagen, and replication index, and improved the lower collagen III/I ratio and the increase in apoptotic index, caused by CNR, compared with sham operation. There were no effects of tadalafil on increased transforming growth factor beta1, inducible nitric oxide synthase and xanthine oxidoreductase levels.

**CONCLUSIONS:** A long-term single daily dose of tadalafil prevented CVOD and the underlying corporal fibrosis in the rat caused by CN damage, as effectively as the previously reported continuous treatment with vardenafil or sildenafil, through a cGMP-related mechanism that appears to be independent of inducible nitric oxide synthase induction.

In addition, we discussed collectively our experimental results with PDE5 inhibitors and its clinical implications in a review article (see paper #4 in the Appendix). We reproduce below the summary of this paper.

**Summary of paper #4 Rationale for phosphodiesterase 5 inhibitor use post-radical prostatectomy: experimental and clinical review**

Erectile dysfunction (ED) is a common complication after radical prostatectomy and results from trauma sustained by the cavernosal nerves. This is a major concern for patients and often affects treatment decisions. The likely mechanism for post-prostatectomy ED is through corporal veno-occlusive dysfunction. There is an increasing amount of evidence to suggest that phosphodiesterase 5 inhibitors (PDE5 inhibitors), when given on a continuous long-term basis, can help to prevent and reverse ED after surgery. In this review article we will examine the pathophysiology of post-prostatectomy ED and discuss the experimental and available clinical evidence for administering PDE5 inhibitors after prostatectomy.

**Bulleted list of key research accomplishments**

We have demonstrated in a rat model of erectile dysfunction subsequent to cavernosal nerve damage during radical prostatectomy for prostate cancer, that:

- Neuropraxia, causes loss of smooth muscle cells and excessive collagen deposition in the penile corpora cavernosa that is responsible for the impaired corporal compliance leading to corporal veno-occlusive dysfunction (CVOD), or corporal venous leakage, the prevalent form of vasculogenic erectile dysfunction
- The spontaneous induction of iNOS, leading to sustained higher levels of nitric oxide and cGMP, is an endogenous antifibrotic mechanism in the corpora cavernosa of this rat model that aims to counteract the pathophysiology of CVOD
- Long-term continuous administration of PDE5 inhibitors immediately after cavernosal nerve damage, to maintain sustained moderately high levels of cGMP, prevents CVOD and the underlying histopathology of the corpora cavernosa

In addition, we believe that our most significant accomplishments so far are that these experimental studies, combined with our similar work in the aged and diabetic rat models:

- have provided the proof of concept for the efficacy of a long-term continuous treatment with PDE5 inhibitors, as opposed to sporadic on demand administration to elicit an erection, to prevent and/or counteract the development of erectile dysfunction and its underlying corporal tissue pathology, after radical prostatectomy
- have provided the theoretical framework to justify the recent FDA approval of daily continuous use of tadalafil (Cialis) for erectile dysfunction, even if this approval is based on the well known vasoactive effects of PDE5 inhibitors without any reference to their potential ability to correct the underlying histopathology; from our results we expect that the patient may become gradually independent from this medication once the normal corporal tissue composition is restored.

**Reportable outcomes**

A. Papers acknowledging this grant (see Appendix)

1. Ferrini MG, Kovanecz I, Sanchez S, Vernet D, Umeh C, Rajfer J, Gonzalez-Cadavid NF. Fibrosis and loss of smooth muscle in the corpora cavernosa precede corporal veno-occlusive dysfunction (CVOD) induced by experimental cavernosal nerve damage in the rat. BJU Int, submitted

2: Kovanecz I, Rambhatla A, Ferrini M, Vernet D, Sanchez S, Rajfer J, Gonzalez-Cadavid N (2008) Long-term continuous sildenafil treatment ameliorates corporal veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats. Int J Impot Res. 2008 Mar-Apr;20(2):202-12.

3. Kovanecz I, Rambhatla A, Ferrini MG, Vernet D, Sanchez S, Rajfer J, Gonzalez-Cadavid N (2008) Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction that occurs after cavernosal nerve resection. BJU Int. Jan;101(2):203-10.

4. Rambhatla A, Kovanecz I, Ferrini M, Gonzalez-Cadavid NF, Rajfer J. Rationale for phosphodiesterase 5 inhibitor use post-radical prostatectomy: experimental and clinical review. Int J Impot Res. 2008 Jan-Feb;20(1):30-4.

B. Abstracts and presentations

1. Ferrini MG, Kovanecz I, Sanchez S, Rajfer J, Gonzalez-Cadavid NF. Time course of corporal veno-occlusive dysfunction (CVOD) development and inducible nitric oxide synthase (iNOS) expression in rat penile tissue after cavernosal nerve damage. J Urol 177 (4): 262-62 Suppl. S, 2007

C. New applications for funding

The following grant applications have been submitted by investigators in this DOD grant using in part results obtained during year 1 of this grant.

1. Modulation of stem cell differentiation in diabetes-related erectile dysfunction (PI: Gonzalez-Cadavid NF). Submitted on 03/17/08 as main research grant in the O'Brien Urology Center at LABioMed Harbor-UCLA, to NIH-NIDDK in response to RFA
2. Repair of cavernosal nerve damage using stem cells and nitric oxide upregulators (PI: Ferrini, MG)**.** Submitted on 03/17/08 as main research grant in the O'Brien Urology Center at LABioMed Harbor-UCLA, to NIH-NIDDK in response to RFA
3. O'Brien Urology Center at LABioMed Harbor-UCLA (Associate Director: Gonzalez-Cadavid NF) Submitted 03/17/08 as a grant to fund a Center based at LABioMed, to NIH-NIDDK in response to RFA

D. Appointments

In part because of the successful outcomes of this grant during the first year, the principal investigator responsible for the LABioMed site, Dr. Monica G. Ferrini has been recruited as full time faculty at Charles Drew University, where she will pursue her own independent research career. Therefore, she has resigned her LABioMed position and her site-PI role in this grant.

A collaborator at LABioMed, Dr. Istvan Kovanecz, has been selected to act as new PI at LABioMed, since he is first author in two of the above papers and coauthor in all of them, and very experienced in animal work and immuno-histochemistry. His biosketch is enclosed in the Appendix. In this way all the animal experiments will continue at LABioMed, where the IACUC has approved our protocols.

## Conclusions

Cavernosal nerve damage, resembling the one caused by radical prostatectomy for prostate cancer, causes loss of smooth muscle cells and excessive collagen deposition in the penile corpora cavernosa. This is responsible for the impaired corporal compliance leading to corporal veno-occlusive dysfunction (CVOD), the prevalent form of vasculogenic erectile dysfunction that develops in many of these patients**.** The spontaneous induction of iNOS, leading to sustained higher levels of nitric oxide and cGMP, is an endogenous antifibrotic mechanism in the corpora cavernosa of this rat model that aims to counteract the pathophysiology of CVOD**.** Long-term continuous administration of PDE5 inhibitors immediately after cavernosal nerve damage, maintaining moderately high levels of cGMP, prevents CVOD

and the underlying histopathology of the corpora cavernosa. This may soon be translated into the clinic, once the appropriate dosing is established, as a treatment to prevent or counteract erectile dysfunction after radical prostatectomy.

## Summary of results

CVOD appeared late (30 days) in the BCNR rats as compared to the sham controls, and exacerbated at 45 days. This functional impairment was increased by continuous oral administration of the iNOS inhibitor L-NIL. In contrast, the SMC/collagen ratio in the BCNR corpora was reduced early (7 days) and bottomed at 30 and 45 days, due in part to the reduction of SMC, presumably caused by an increase in apoptosis peaking at 3 days but remaining high thereafter. Cell proliferation also peaked at 3 days but then normalized. nNOS was reduced early (3-7 days) and disappeared at 30 days, whereas eNOS was not affected. iNOS was induced at day 3, and steadily increased to high levels peaking at 30 days.

The moderate CVOD developing in the BCNR rat most likely results from the early loss of corporal SMC by the neuropraxia-induced apoptosis and the associated fibrosis, which the early cell replication response cannot counteract. The absence of eNOS decrease suggests that the endothelium is not affected to the same extent. The time course of iNOS induction and the exacerbation of CVOD and fibrosis by L-NIL support an antifibrotic role for iNOS.

PDE5 inhibitors, namely vardenafil, sildenafil, and tadalafil: 1) prevented the 30% decrease in the smooth muscle cell/collagen ratio, and the 3-4-fold increase in apoptosis and reduction in cell proliferation, and partially counteracted the increase in collagen, seen with both UCNR and BCNR; and 2) normalized the CVOD, measured by dynamic infusion cavernosometry, induced by both BCNR and UCNR. The long-term inhibition of iNOS activity exacerbated corporal fibrosis and CVOD in the BCNR rats, but sildenafil functional effects were not affected by L-NIL. These data suggest that the salutary effects of continuous long-term PDE5 inhibitors on erectile function post-cavernosal nerve resection involve their ability to prevent the alterations in corporal histology induced by cavernosal nerve damage, in a process apparently independent from endogenous iNOS induction.

## References

They are listed in the papers enclosed in the Appendix

## Appendices

They include:

1) The manuscript of paper 1 and the downloaded publications for papers 2-4
2) The biographical sketches of Drs. Gonzalez-Cadavid, Ferrini, and Kovanecz

# FIBROSIS AND LOSS OF SMOOTH MUSCLE IN THE CORPORA CAVERNOSA PRECEDE CORPORAL VENO-OCCLUSIVE DYSFUNCTION (CVOD) INDUCED BY EXPERIMENTAL CAVERNOSAL NERVE DAMAGE IN THE RAT.

**Ferrini M.G[1,2,3], Kovanecz I[1,2], Sanchez S[1], Umeh C[1], Rajfer J[1,2], Gonzalez-Cadavid NF[1,2,3]**

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**Running Title:** sequence of fibrosis and CVOD after cavernosal nerve damage

**Key words:** erectile dysfunction, nerve sparing, radical prostatectomy, penis, nitric oxide; cGMP, collagen, inducible nitric oxide synthase, apoptosis

**Abbreviations: ASMA:** $\alpha$-smooth muscle actin; **BCNR:** bilateral cavernosal nerve resection; **CVOD:** corporal veno-occlusive dysfunction; **DIC**: dynamic infusion cavernosometry; **GAPDH:** glyceraldehide-3-phosphate dehydrogenase; **ICP**: intracavernosal pressure; **iNOS:** NOS II, inducible nitric oxide synthase; **PCNA**: proliferating cell nuclear antigen; **QIA:** quantitative image analysis**; SMC:** smooth muscle cells

**Received:** 03/24/08

**ABSTRACT**

**Background and Objectives**. CVOD is present in the rat at 45 days after bilateral cavernosal nerve resection **(BCNR)**, a model for erectile dysfunction in men subsequent to radical prostatectomy. This is associated with a loss of smooth muscle cells **(SMC)** and fibrosis of the penile corpora cavernosa, accompanied by the expression of inducible nitric oxide synthase **(iNOS)** which is assumed to be vasculoprotective and antifibrotic. We have now studied the temporal relationship of these processes to determine whether CVOD occurs after corporal fibrosis and SMC loss and whether iNOS induction increases steadily throughout.

**Methods**. Fisher 344 male rats were subjected to either BCNR or sham operation, and then to cavernosometry after 1, 3, 7, 15, 30, and 45 days (n=8/group). Penile shaft tissue sections were subjected to Masson trichrome, immunodetection for alpha smooth muscle actin **(ASMA)** as SMC marker, iNOS, neuronal NOS (nNOS), endothelial NOS (eNOS), proliferating cell nuclear actin **(PCNA)** for cell proliferation, and TUNEL for apoptosis, followed by quantitative image analysis. Quantitative western blot measured some of these markers in tissue homogenates

**Results.** CVOD appeared late (30 days) in the BCNR rats as compared to the sham controls, and exacerbated at 45 days, and even more by L-NIL. In contrast, the SMC/collagen ratio in the BCNR corpora was reduced early (7 days) and bottomed at 30 and 45 days, due in part to the reduction of SMC, presumably caused by an increase in apoptosis peaking at 3 days but remaining high thereafter. Cell proliferation also peaked at 3 days but then normalized. nNOS was reduced early (3-7 days) and disappeared at 30 days, whereas eNOS was not affected. iNOS was induced at day 3, and steadily increased to high levels peaking at 30 days.

**Conclusions**. CVOD develops in the BCNR rat as a result of the early loss of corporal SMC by the neuropraxia-induced apoptosis, which the initial cell replication response cannot counteract, followed by fibrosis. The absence of eNOS decrease suggests that the endothelium is not

considerably affected. The time course of iNOS induction and the previous demonstration of CVOD and fibrosis increase by the iNOS inhibitor L-NIL support an antifibrotic role for iNOS.

**INTRODUCTION**

Despite the use of nerve-sparing surgical techniques during radical prostatectomy, the cavernosal nerves still appear to be somewhat susceptible to injury during the surgical procedure as evidenced by persistent and relatively high rates of erectile dysfunction in the immediate post operative period following these nerve sparing techniques **(1-4)**. The primary cause of this impotence post-RP is corporal veno-occlusive dysfunction **(CVOD)** or venous leakage **(5-8).** It becomes manifest whenever there is a decrease in the content of corporal smooth muscle cells **(SMC).** When this occurs, the remaining corporal smooth muscle mass is unable to achieve sufficient relaxation thereby preventing the development of a high intracorporeal pressure which is necessary for the passive occlusion of the veins egressing from the corporal bodies as they traverse underneath and then through the tunica albuginea of the penis.

We have previously demonstrated in the rat, in a model of cavernosal nerve resection, that CVOD is detectable at 45 days after surgery **(9-12).** This functional impairment was associated with a decrease in the SMC mass and an increase in collagen content in the corporal tissue. Our group was also able to demonstrate long-term continuous oral administration of a PDE5 inhibitor in rats, such as vardenafil **(11),** sildenafil **(10)** and tadalafil **(9)** prevents this neuropraxia-induced CVOD, presumably due to the antifibrotic effects of the PDE5 inhibitors, and also to the preservation of the corporal SMC mass by up-regulation of SMC replication.

In our experiments we also found an increase in the expression of the inducible nitric oxide synthase **(iNOS)** following BCNR, which was further stimulated by vardenafil, but not by sildenafil and tadalafil, suggesting that neuropraxia may upregulate iNOS **(9-11).** The resulting

steady output of nitric oxide and cGMP from the tissue may in turn contribute to the antifibrotic and vasculoprotective effects of PDE5 inhibitors that inhibit cGMP breakdown.

However, even though it has been well established that CVOD develops after BCNR, it is not known when this impairment starts to manifest, whether it is preceded by the loss of SMC and fibrosis, and how does it relate to the process of iNOS induction as a putative antifibrotic mechanism. The aim of this study was to determine in a time course the development of the histological and functional changes that occur after BCNR, in order to establish the time frame to initiate the treatment with the PDE5 inhibitors after cavernosal nerve damage and to clarify the sequence of iNOS induction in relation to these processes .

## MATERIALS AND METHODS

### *Animal treatments*

Five month-old male Fisher 344 rats (Harlan Sprague–Dawley, San Diego, CA) were randomly divided into sham operated and BCNR groups. Animals were sacrificed at 1, 3, 7, 15, 30 and 45 days after surgery (n=8 each group). BCNR was performed as described **(9-11)**. In the sham-operated group both cavernosal nerves were identified but not resected. In BCNR, the main cavernosal nerves and ancillary branches were resected by removing a 5-mm segment. All animal experiments were approved by the IACUC at our institution.

### *Dynamic Infusion Cavernosometry (DIC)*

Cavernosometry was performed as previously described **(9-11,13).** Briefly, basal intracavernosal pressure **(ICP)** was recorded, and papaverine (20 mg/ml) was administered through a cannula into the corpora cavernosa. The ICP during tumescence was recorded as “ICP after papaverine”. Saline was then infused through another cannula, increasing infusion rate by 0.05 ml/min every 10 seconds, until the ICP reached 80 mmHg ("maintenance rate").

The “drop rate” was determined by recording the fall in ICP within the next 1 minute after the infusion was stopped.

***Histochemistry and immunohistochemistry***

After cavernosometry, animals were sacrificed and the skin-denuded penile shafts were fixed overnight in 10% formalin, washed, and stored in alcohol 70 % at 4C until processed for paraffin embedded tissue sections (5 um). Adjacent sections were used for **(9-11)**: a) Masson trichrome staining for collagen (blue) and SMC (red)**;** c) immunodetection with: c1) monoclonal antibodies against α-smooth muscle actin **(ASMA)** as a SMC marker (Sigma kit, Sigma Diagnostics, St Louis, MO) and proliferating cell nuclear antigen **(PCNA)** as marker of cell proliferation (Chemicon, Temecula, CA); c2) polyclonal antibody against iNOS **(14)** (Calbiochem, La Jolla, CA); c3) monoclonal antibody against eNOS **(15)** (BD pharmaceutical, ***location??);*** c4) monoclonal antibody against nNOS **(16)** ***(source???).*** The specificity of the antibodies was validated by western blot.

Sections were then incubated with biotinylated anti-Mouse IgG (ASMA PCNA, eNOS, nNOS) or biotinylated anti-Rabbit IgG (iNOS), respectively, followed by ABC complex (Vector labs, Temecula, CA) and 3,3’diaminobenzidine (Sigma) (PCNA and iNOS), or with the ASMA Sigma kit (ASMA) and 3-amino-9-ethylcarbazole**.** TUNEL assay was performed as described **(9-11)** applying the Apoptag peroxidase detection assay (Chemicon), with TdT enzyme and anti-digoxigenin-conjugated peroxidase, and 3,3’diaminobenzidine/H2O2. Sections were counterstained with hematoxylin. Negative controls in the immunohistochemical detections were done by replacing the first antibody with IgG isotype. The negative control for TUNEL was by substituting buffer for the TdT enzyme. Testicular tissue sections were used as positive control.

***Quantitative image analysis***

Quantitative image analysis **(QIA)** was performed by computerized densitometry using the ImagePro 4.01 program (Media Cybernetics, Silver Spring, MD), coupled to an Olympus

BHS microscope equipped with an Olympus digital camera **(9-11).** For Masson staining, 40x magnification pictures of the penis comprising half of the corpora cavernosa were analyzed for SM (stained in red) and collagen areas (stained in blue), and expressed as SMC/collagen ratios. For ASMA and iNOS staining, only the corpora cavernosa was analyzed in a computerized grid and expressed as % of positive vs. total area of the corpora cavernosa. For PCNA and TUNEL determinations, the number of positive cells at 400x was counted and results were expressed as a % of positive cells/total cells in the corpora cavernosa. In all cases, two fields at 40x, or 8 fields at 400x, were analyzed per tissue section, with at least 4 matched sections per animal and 8 animals per group.

***Western blot analysis***

Penile tissue homogenates (100 mg tissue) were obtained in T-PER (PIERCE, Rockford, IL) and protease inhibitors (3 μM leupeptin, 1 μM pepstatin A, 1mM phenyl methyl sulfonyl fluoride), and centrifuged at 10,000 g for 5 min. Supernatant protein (30-50 μg) were subjected to western blot analyses **(17-19)** by 7-10 % Tris-HCl polyacrylamide gel electrophoresis (PAGE) (Bio-Rad, Hercules, CA) in running buffer (Tris/Glycine/SDS). Proteins were transferred overnight at 4ºC to nitrocellulose membranes in transfer buffer (Tris/glycine/methanol). Next day, the non-specific binding was blocked by immersing the membranes into 5% non-fat dried milk, 0.1% (v/v) Tween 20 in PBS for 1hour at room temperature. After several washes with washing buffer (PBS Tween 0.1%), the membranes were incubated with the primary antibodies for 1 hour at room temperature monoclonal antibodies were as follows: a) ASMA, as above (1/1000) (Calbiochem, La Jolla, CA); b) glyceraldehide-3-phosphate dehydrogenase **(GAPDH)** (1/10,000) (Chemicon International, Temecula, CA); and c) PCNA (Chemicon International).The washed membranes were incubated for 1 hour at room temperature with 1/3,000 dilution (anti-mouse), followed by secondary antibody linked to horseradish peroxidase. After several washes, the immunoreactive bands were visualized using the ECL plus western blotting

chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). The densitometry analysis of the bands was done with Image J (NIH, Bethesda, MD). A positive control was run throughout all gels for each antibody to standardize for variations in exposures and staining intensities. Negative controls were performed omitting the primary antibody. Band intensities were determined by densitometry and corrected by the respective intensities for a housekeeping protein, glyceraldehyde phosphate dehydrogenase (GAPDH), upon reprobing.

***Statistical analysis***

Values were expressed as Mean ± SEM. The normality distribution of the data was established using the Wilk-Shapiro test. Multiple comparisons were analyzed by a two factors (time and treatment) analysis of variance (two way ANOVA) , followed by post-hoc comparisons with the Bonferroni test, according to the GraphPad Prism V 4.1. Differences were considered significant at $P<0.05$.

**RESULTS**

***CVOD caused by BCNR-induced neuropraxia is preceded by the development of fibrosis by collagen deposition in the corpora cavernosa***

DIC was performed at 1, 3, 7, 14, and 30 days after cavernosal nerve injury and just prior to sacrifice, to evaluate the time period of CVOD appearance. Values for the 45 days time period for this determination, as well for the other assays, are included in this and subsequent figures as reference, and were taken from one of our previous papers with identical sets of BCNR- and sham-operated rats **(11).** However, in all the subsequent figures, the representative micrographies for 14 and 45 days are omitted to reduce space. **Figure 1 (top)** shows that peak ICP following papaverine was not affected by BCNR for at least the first two weeks, in comparison to sham-operated animals, and that it was detected only in the rat groups

corresponding to 30 and 45 days after surgery. A similar situation occurred with the determination of drop rates upon saline infusion, which at the last two periods increased two-fold..

The smooth muscle and collagen contents were determined in cross-sections of the penile corpora cavernosa by three methods. First, Masson trichrome staining was applied to estimate the relative SMC/collagen ratio. **Fig. 2, top left,** shows that there are no visual changes in the staining for collagen and smooth muscle on representative micrographs from the sham operated group over selected periods of the same time course. An expected intensification of collagen staining in the BCNR group **(right )** was first detected at 7 days after surgery and this process was increased at 30 days. Quantitative image analysis **(bottom)** shows that the SMC/collagen ratios first decreased significantly in the BCNR rats at 7 days, and values were as low as 30% of those in the sham groups by 30 and 45 days after surgery. The red staining of the SMC was easily differentiated from the red blood cells, so the latter was not considered in the quantitative determination..

The second procedure estimated directly SMC content based on the immuno-histochemical determination of ASMA, an accepted marker of SMC in the corpora cavernosa**. Fig. 3, A top** shows a considerable reduction with time in ASMA staining in the BCNR group as compared to the sham group, starting to decline as early as 3 days after surgery, and reaching the lowest level at 30 and 45 days. The respective reductions in ASMA content determined by quantitative image analysis **(bottom)** were 40, 76%, and 78% **.** The expression of ASMA in the sham-operated group remained unchanged throughout the experiment. Finally, a third procedure, western blot analysis of ASMA expression in homogenates of penile shaft tissue **(B),** confirmed these results, except that the decreases at 3, 30 and 45 days were smaller (24, 46, and 50%, respectively). Collectively, these results suggest that the corporal fibrosis induced by BCNR precede, as expected, the functional impairment of vasculogenic erectile response, and that the earliest event is smooth muscle loss rather than collagen deposition. This is based on

the fact that the reduction in ASMA + cells is rather considerable at a period (3 days) when the SMC/collagen ratio has only slightly decreased.

***BCNR also causes the expected loss of nNOS nerves without affecting the eNOS present in the endothelium, and this is accompanied by a remarkable stimulation of iNOS induction,***

BCNR is assumed to cause the progressive loss of cavernosal nerve terminals distal to the 3-5 mm resection, and this should impact the overall content of nNOS in the corpora cavernosa. That this is the case was shown by immunohistochemistry with an antibody selective for nNOS that does not cross-react with eNOS and iNOS. **Fig. 4 top** shows that at 1 day there was already a decrease of nNOS staining in the BCNR rats as compared to the sham-operated animals at a nerve terminal in the corpora corresponding to the cavernosal nerve, that intensified at 3 days and persisted throughout. Because the decrease in staining intensity was so evident, no quantitative determination was deemed necessary to corroborate the visual inspection. In contrast, no changes were appreciable in the immunohistochemical detection of eNOS, that was constrained to the endothelium lining of the corpora cavernosa lacunar spaces or cisternae **(Fig. 4 bottom).** This was confirmed by quantitative image analysis.

In an even more marked contrast to nNOS decrease, iNOS immunostaining in the corpora of the BCNR rats started to increase by 10-fold at day 3, and continued to remain high throughout, while it was very low in the shaft-operated animals at all time periods **(Fig. 5 top)**. The quantitative determination indicated that iNOS expression reached a peak at 30 days with an over 50-fold increase over the control value in the sham animals **(bottom).**

***The reduction in SMC occurring after BCNR is due to an early peak of apoptosis that initially is compensated by increased cell proliferation but later on predominates over this process***

TUNEL immunodetection assay revealed that at 1 day, and particularly at 3 days BCNR caused a considerable increase of the basal level of corpora cavernosa cell apoptosis observed in the sham-operated animals, that appeared to decline thereafter **(Fig. 6 top).** The quantitative determination confirmed that the peak occurred at 3 days, with a 5-fold increase in the apoptotic index in the BCNR animals, and this was followed by a gradual reduction, but still showing an over 2-fold higher apoptotic index at 45 days after BCNR **(bottom),**

Remarkably, the initial stages subsequent to BCNR, at 1 and 3 days, presented with an intensification of cell proliferation as compared with sham-operated animals, denoted by immunohistochemistry for PCNA, but subsequently this was reduced considerably, apparently to basal levels **(Fig. 7 top)**. Quantitative image analysis showed that, as in the case of apoptosis, the cell proliferation peak occurred at 3 days, with a similar 5-fold increase in PCNA staining, decreasing thereafter. At 30 days, and particularly 45 days, values in BCNR were lower than in the control sham-operated animals **(middle)**. Because of the initial stimulation of cell replication, the ratio between the proliferation and apoptotic indexes in the corpora **(bottom)** remains around a value of 1 until 7 days after BCNR, with no significant differences between BCNR and sham rats. However, both at 30 and 45 days there is a considerable reduction due to the predominance of cell death over proliferation. This agrees with the time course for SMC content in Fig. 3.

The western blot analysis of PCNA expression in total penile shaft homogenates presented on **Fig. 8** confirmed the decrease in PCNA staining in the corpora cavernosa of BCNR rats observed by immunohistochemistry in tissue sections in the preceding figure. However, the levels of PCNA in the homogenates of the penile shaft were inconsistently high at the two earliest periods, probably reflecting the presence of tunical and corpus spongiosum tissue (not considered in the analysis of the tissue sections) where cell replication was possibly stimulated by the sham operation.

**DISCUSSION**

The current results clarify the sequence of events that lead to fibrosis and loss of SMC in the corpora cavernosa and the resulting CVOD after cavernosal nerve damage, that we have previously defined as occurring 45 days after BCNR in the rat model **(9-11).** It is evident that as early as 1 day after neuropraxia there is an increase of programmed cell death that peaks at 3 days, thus confirming results of other groups **(20-23)**, but that is initially compensated by an equally considerable increase in cell proliferation that had not been so far reported. Therafter, cell proliferation, by drastically declining already at 7 days, becomes insufficient to counteract the much slower decline in apoptosis. As a result, the inbalance between both processes manifests at 30 days, agreeing with the earliest period where there is a net loss of SMC. Since the SMC/collagen ratio first falls down significantly, and rather drastically, at 7 days after BCNR, but the SMC content decreased much earlier, at 3 days, coinciding with the apoptosis peak, one must conclude that collagen deposition is intensified after the SMC loss, and that therefore the reduction of the cellular compartment precedes fibrosis. It is the net loss of SMC what appears to trigger the first manifestation of CVOD that occurs 30 days after BCNR.

On the other hand, the reduction of nitrergic nerves in terminals that are clearly distinguishable from the dorsal nerve and may be ascribed topologically to the cavernosal nerve, are evident already at 1 day after BCNR, thus suggesting that Wallerian nerve degeneration exacerbated throughout the 45 day-period may be responsible for the changes observed in the corpora cavernosa SMC. Significantly, the lack of changes in the content of eNOS suggests that the endothelium is not considerably affected by BCNR**,** a critical result that indicates that: a) endothelial dysfunction is not elicited by neuropraxia and is not involved in CVOD, that appears to result from corporal SMC loss and fibrosis; and b) in the absence of nNOS, eNOS cannot per se produce sufficient nitric oxide as to sustain the papaverine-induced production of cGMP caused by the unspecific PDE inhibition exerted by the drug **(24).**

Perhaps the most intriguing observation is the time course of iNOS induction by BCNR, that seems to follow nNOS decrease in the nitrergic nerves but peaks at 30 day***s,*** long after apoptosis has reached a maximum at 3 days, thus ruling out the possibility that this cell death is triggered by nitric oxide from iNOS, a compound that is usually considered as pro-apoptotic **(25,26)**. However, there is evidence that nitric oxide can be anti-apoptotic according to tissue and physiological conditions **(27).** The alternative that this sustained increase of iNOS expression would be what reduces the compensatory cell proliferation in the corpora after BCNR, based on the fact that both nitric oxide and cGMP are considered to be antiproliferative for the SMC in the arterial media **(28),** appears to be ruled out by our previous results with L-NIL, an inhibitor of iNOS activity **(10,14)**. At least at 45 days after BCNR, a steady iNOS inhibition by daily oral L-NIL significantly reduced the SMC/collagen ratio***,*** thus suggesting that iNOS is acting protecting the SMC, and this may occur by either the increase in cell proliferation that we have previously postulated **(10)** or the inhibition of apoptosis or both***.*** This would be in agreement with the cardioprotective effects of nitric oxide, cGMP, and iNOS on cardiomyocytes during ischemia reperfusion pre- or post-conditioning **(29-31).**

Our view regarding the protective role of iNOS induction in the corpora after cavernosal nerve damage is based on previous studies where we proposed that sustained iNOS expression, and hence enhanced but controlled levels of nitric oxide and cGMP synthesis, to be an antifibrotic mechanism of defense aimed to inhibit excessive collagen synthesis in the context of acute or chronic tissue injury in the vascular system **(32)**. We based our claims on: a) iNOS gene transfer to the penile tunica albuginea inhibited the development of a Peyronie's disease-like fibrotic plaque in the rat **(33)**; b) long-term continuous treatment with L-NIL intensified this plaque **(34)** and the arterial media fibrosis associated with aging in the rat **(14)**; c) a similar treatment in the BCNR rats for 45 days reduced the SMC/collagen content and intensified CVOD **(10)**; d) iNOS knock out mice had an increased collagen deposition in the penile corpora cavernosa with aging **(35)**, and adult animals displayed intensified liver and

kidney fibrosis caused by either fat-rich diet or urether ligation (36,37); and e) the long-term continuous administration of nitric oxide generators and PDE5 inhibitors increased the SMC/collagen ratio and reduced collagen deposition in the corpora cavernosa in UCNR, BCNR, and aging, and in the Peyronie's like plaque in the rat **(9-12,38-40)**.

Our previous results with L-NIL, although not directly pertinent to collagen deposition, but also showing an intensification of CVOD **(10)** support our view of iNOS induction after BCNR being protective for the corpora cavernosa. However, since CVOD and fibrosis do develop in BCNR despite the steady iNOS production, this process does not appear to suffice to counteract the factors that trigger "corporal dystrophy" (fibrosis and SMC loss), a term that we propose as analogous to skeletal muscle dystrophy.

This leads to the fundamental question on which are these factors triggered by neuropraxia that cause corporal SMC dystrophy. The most likely is the interruption of the secretion of neurotrophins which in addition to their effects on neural tissue are postulated to stimulate smooth muscle hyperplasia, particularly in the respiratory airways and intestine **(41,42),** a depletion that may cause the down-regulation of SMC proliferation triggered by a spontaneous defense mechanism against neuropraxia**.** Conversely, the induction of cytokine release, mainly TNFα and TGFβ1, that are pro-apoptotic and fibrotic factors and activate the proteasome ubiquitin proteolytic pathway, is a feature of Wallerian degeneration **(43),** and it underlies, at least in part, the skeletal muscle atrophy subsequent to denervation **(44,45).** However, the lack of neuromotor discharge and activity may also be an essential factor in this atrophy.

Irrespective of the mechanism that triggers fibrosis and SMC loss subsequent to cavernosal nerve damage, three things became obvious through this work. First, that is the early histopathological impairment in the corpora smooth muscle that leads to the functional CVOD, and that therefore in the clinical setting an early therapeutical intervention to reduce apoptosis or sustain the initial cell proliferation response, would be warranted, e.g., immediately

after radical prostatectomy. Second, that since iNOS induction appears to be an antifibrotic and smooth muscle protective tissue endogenous reaction, the early therapy may be based on pharmacological agents that mimic this process, such as the continuous long-term administration of PDE5 inhibitors we have studied in rats **(9-12,40),** or of nitric oxide generators **(38,39),** or both. This modality may be accompanied with neurotrophin administration to restore the anabolic signals to the smooth muscle that endogenous neurotrophic factors are no longer mediating. Third, that although axonal regeneration may not occur upon the nerve resection approach that characterized the BCNR model in the rat, it may gradually develop in the less drastic nerve damage caused by nerve sparing procedures during radical prostatectomy, and may even be stimulated by some of these treatments. This would ultimately favor the repair of the corporal fibrosis and loss of smooth muscle, and therefore CVOD, caused by cavernosal nerve damage.

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**LEGENDS TO FIGURES**

**Figure 1. Time course of effect of bilateral cavernosal nerve resection on the erectile function of the rat measured by pharmacological and infusion cavernosometry.** Top: Response of the intracavernosal pressure to papaverine; Bottom: Response of the intracavernosal pressure to the interruption of saline infusion. SHAM: sham-operated animals; BCNR: animals subjected to nerve resection and killed at 1,3,7,15 30 and 45 days after surgery.*: p<0.05; **: p<0.01; ***P<0.001

**Figure 2. Time course of the effect of bilateral cavernosal nerve resection on the smooth muscle/collagen ratio in the rat corpora cavernosa.** Penile corpora cavernosa tissue sections from the rat groups presented on Figure 1, were stained with Masson trichrome. Top: representative pictures (200X, Bar=50 µm). Bottom: quantitative image analysis. SHAM: sham-operated animals; BCNR: animals subjected to nerve resection killed at 1,3,7 and 30 days after surgery. ***: p<0.001

**Figure 3. Time course of the effect of bilateral cavernosal nerve resection on the smooth muscle cell content in the rat corpora cavernosa.** Penile corpora cavernosa sections adjacent to those presented on Fig. 2 were immunostained for ASMA as a smooth muscle cell marker. Top: 40X, Bar=50 µm). Middle: quantitative image analysis.. ***: p<0.001

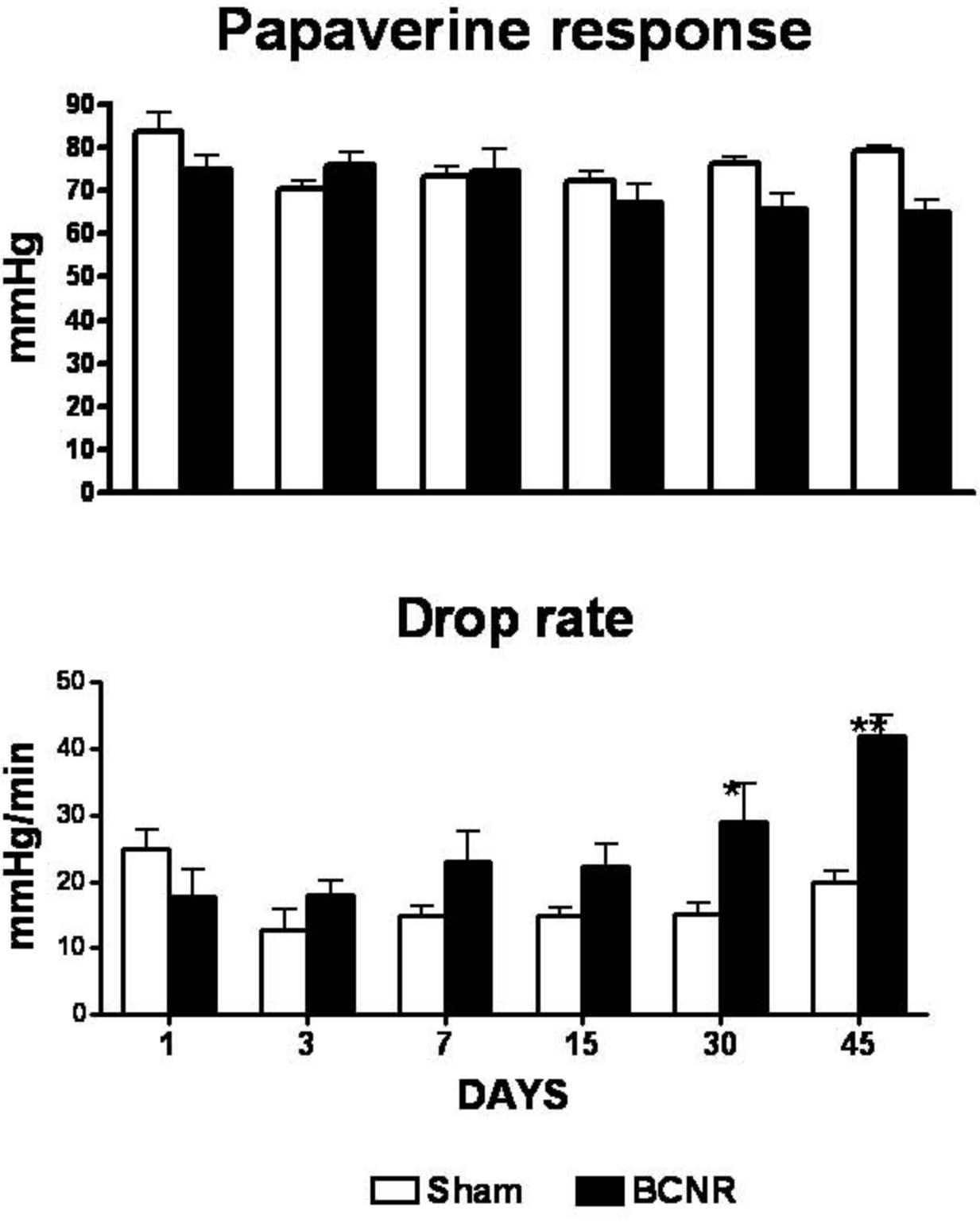
**Figure 4. Time course of nNOS expression after nerve resection in cavernosal nerve terminals and of eNOS in the corporal enothelium.** Penile sections sections adjacent to those presented on Fig. 2 were immunostained for nNOS. Magnification: 400X, Bar=50 µm). Sections adjacent to those presented on Fig. 2 were immunostained with an e-NOS antibody. The expression of eNOS is not altered by nerve resection. Top: 400X, Bar=50 µm). Bottom: quantitative image analysis.

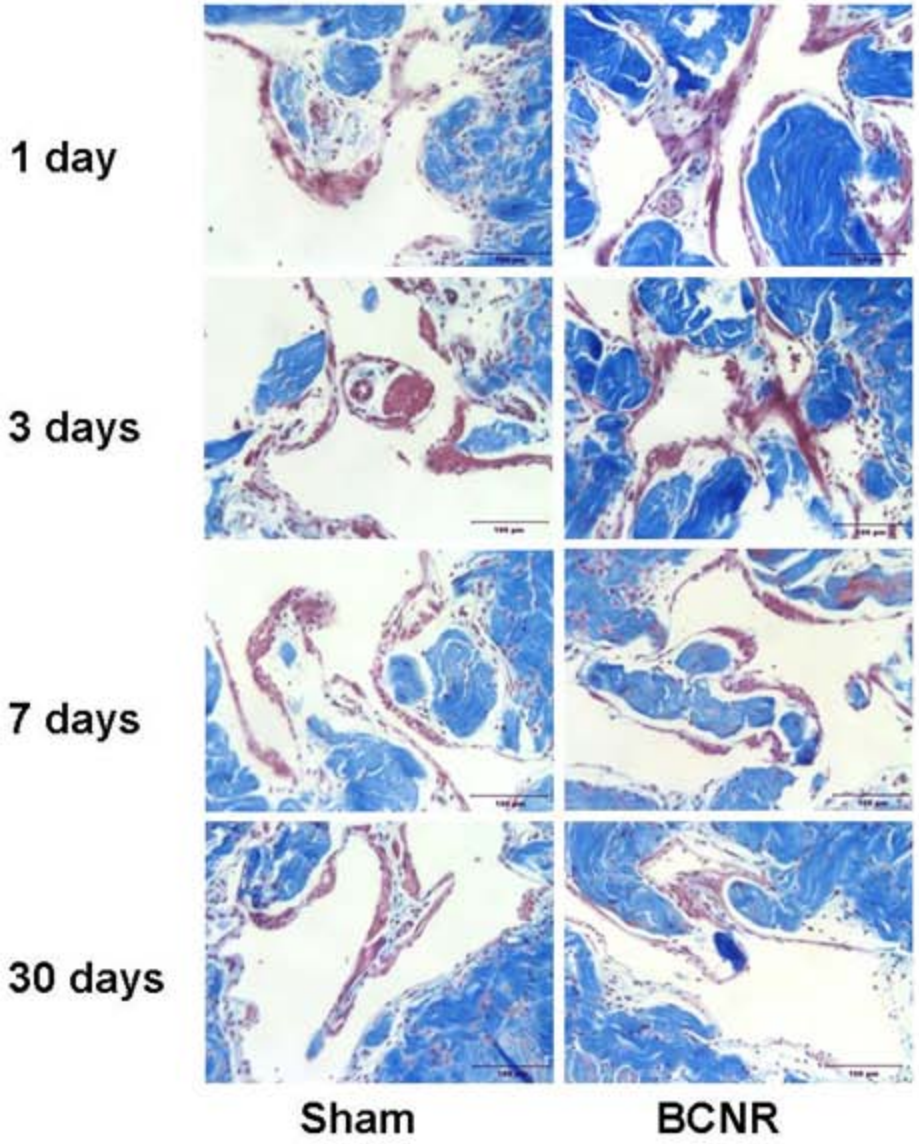
**Figure 5. Time course on the effect of bilateral cavernosal nerve resection on the expression of iNOS in the penile corpora cavernosa.** Penile corpora cavernosa sections adjacent to those presented in Fig 2 were subjected to immunodetection for iNOS. Top: representative pictures (200X, Bar=50 μm). Bottom: quantitative image analysis. ***p<0.001.

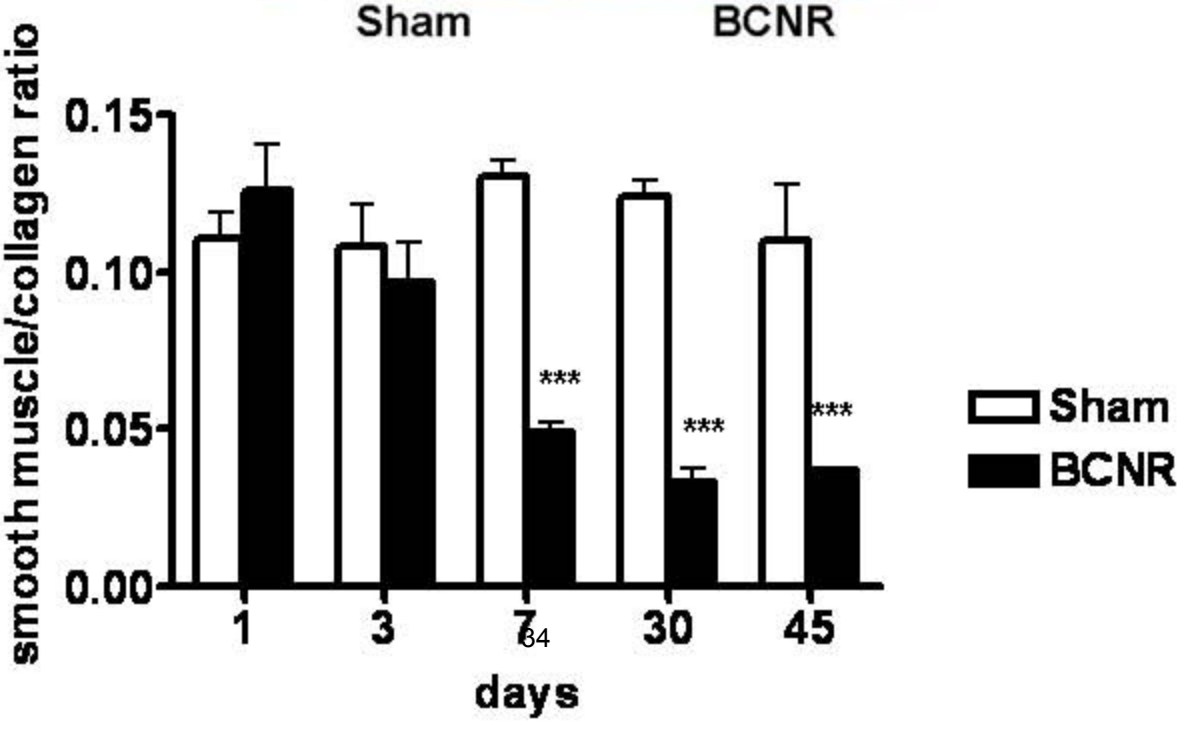
**Figure 6. Time course of the effect of bilateral cavernosal nerve resection on the apoptotic index in the rat corpora cavernosa.** Penile corpora cavernosal sections adjacent to those presented on the preceding figures were subjected to TUNEL staining. Top: representative pictures fo apoptosis by TUNEL (400X). Bar iindicates the apoptotic cells in the corpora cavernosa. Bottom: QIA for TUNEL ***P<0.001

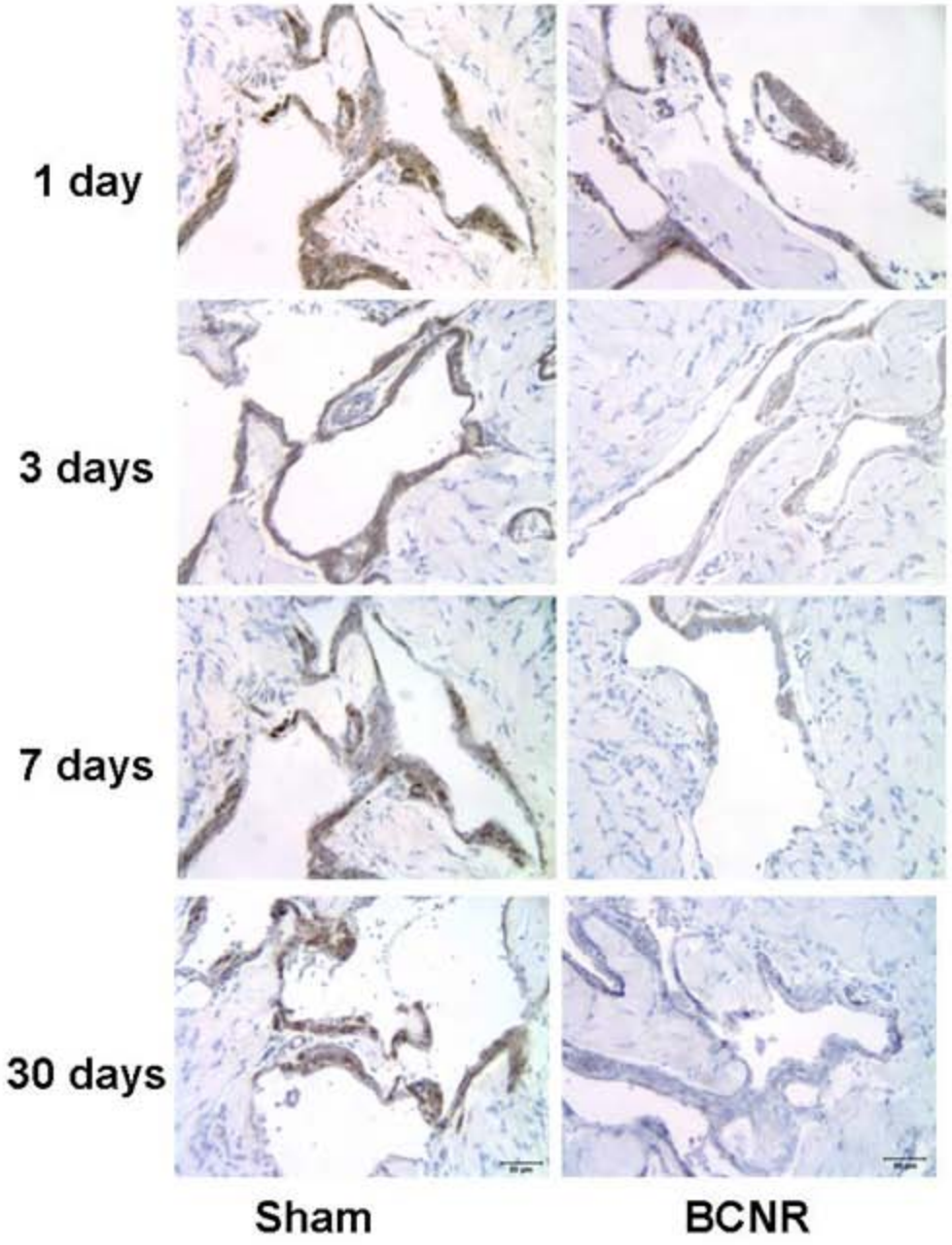
**Figure 7: Time course of the effect of bilateral cavernosal nerve resection on cell proliferation in the corpora cavernosa.** Penile corpora cavernosal sections adjacent to those presented on the preceding figures were subjected to PCNA staining. Top: representative pictures for PCNA (400X). Bottom left : QIA for PCNA; Bottom right: The ratio between the total area occupied by cells undergoing cell replication (PCNA+) and the apoptotic index obtained above was established for each animal, and then used to calculate means+/-SEM. * p:<0.05; **: p<0.01; ***p<0.001

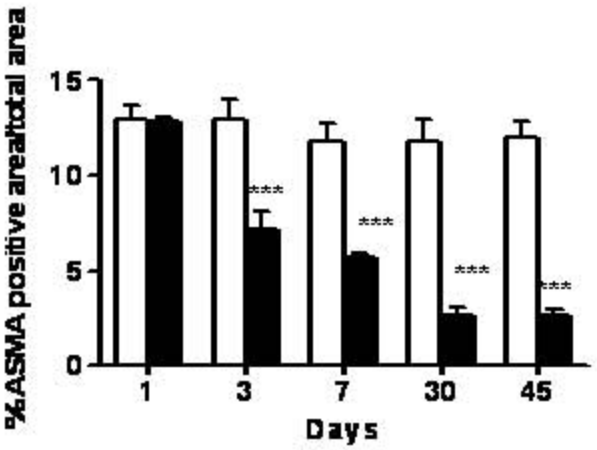
**Figure 8: Corroboration of the PCNA immunostaining by western blot**. Homogenates from corpora cavernosa tissue were subjected to western blot analysis with the same antibody used for figure 8. ** P<0.01

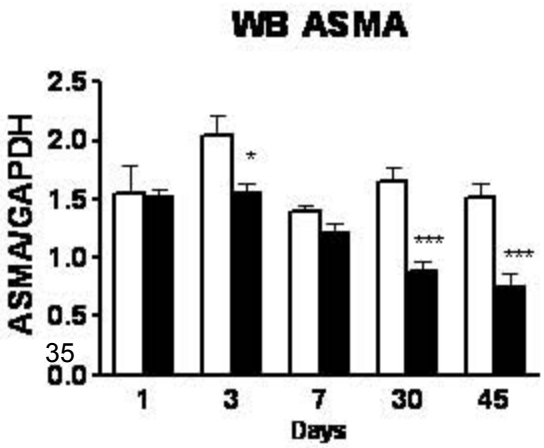

Papaverine response
mmHg
90
80
70
60
50
40
30
20
10
0
Drop rate
mmHg/min
50
40
30
20
10
0
*
**
1
3
7
15
30
45
DAYS
Sham
BCNR


1 day
3 days
7 days
30 days
Sham
BCNR

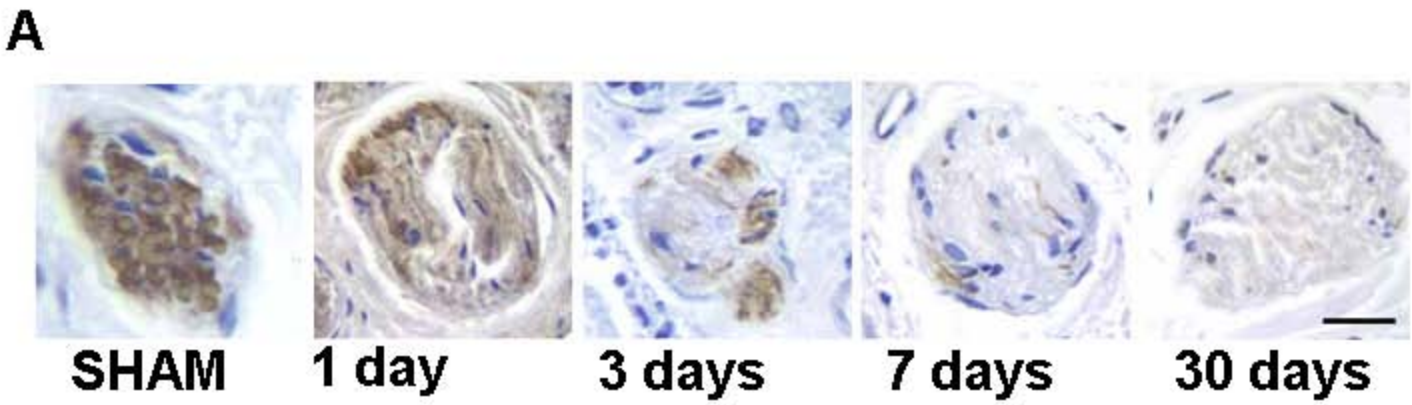

smooth muscle/collagen ratio
0.15
0.10
0.05
0.00
1
3
7
30
45
days
***
***
***
Sham
BCNR

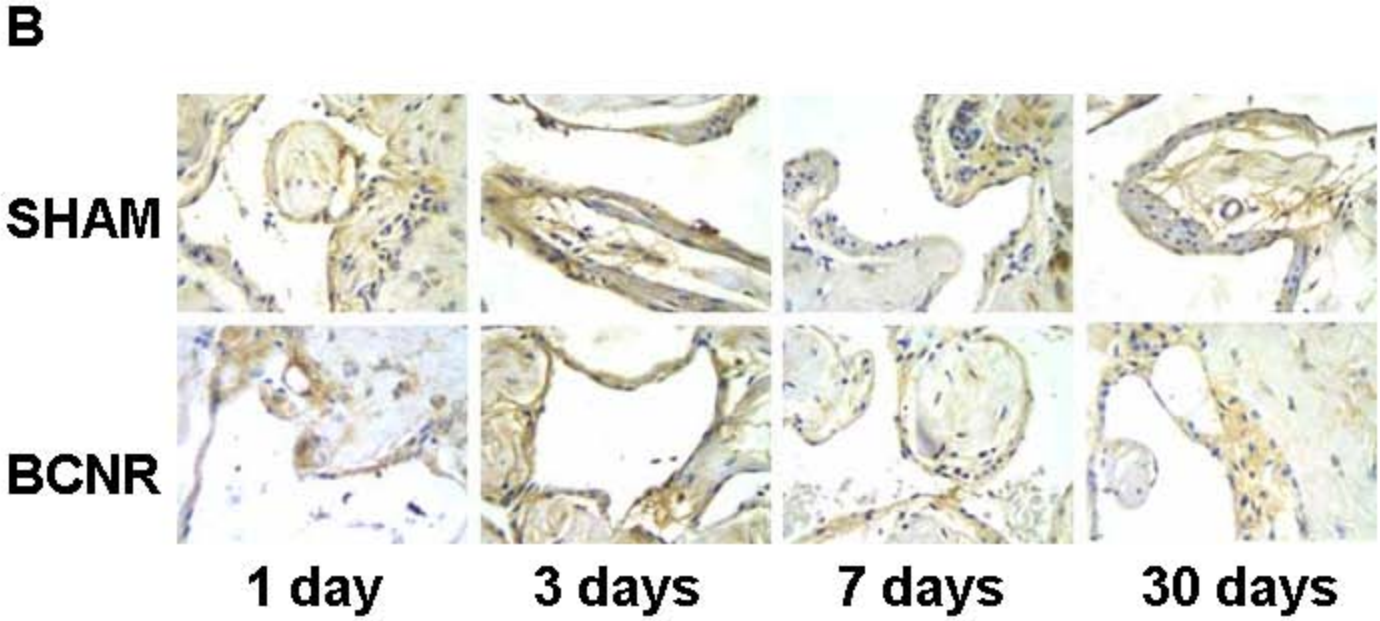

1 day
3 days
7 days
30 days
Sham
BCNR

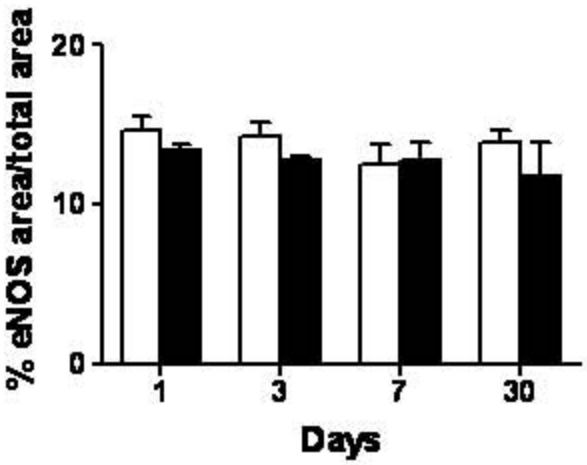

%ASMA positive area/total area
15
10
5
0
***
***
***
***
1
3
7
30
45
Days

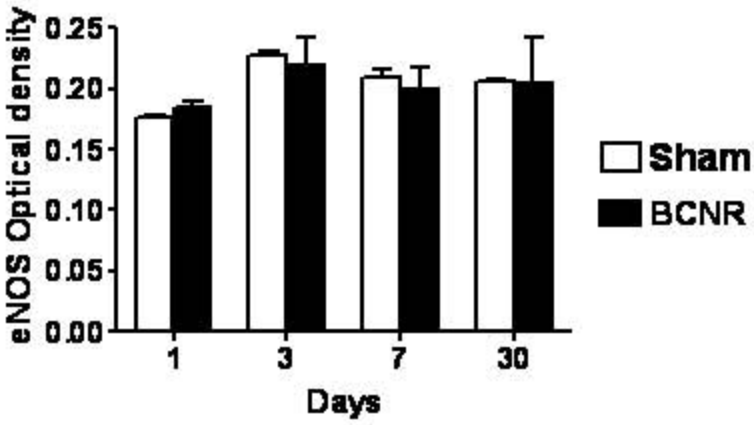

WB ASMA
ASMA/GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
*
***
***
1
3
7
30
45
Days

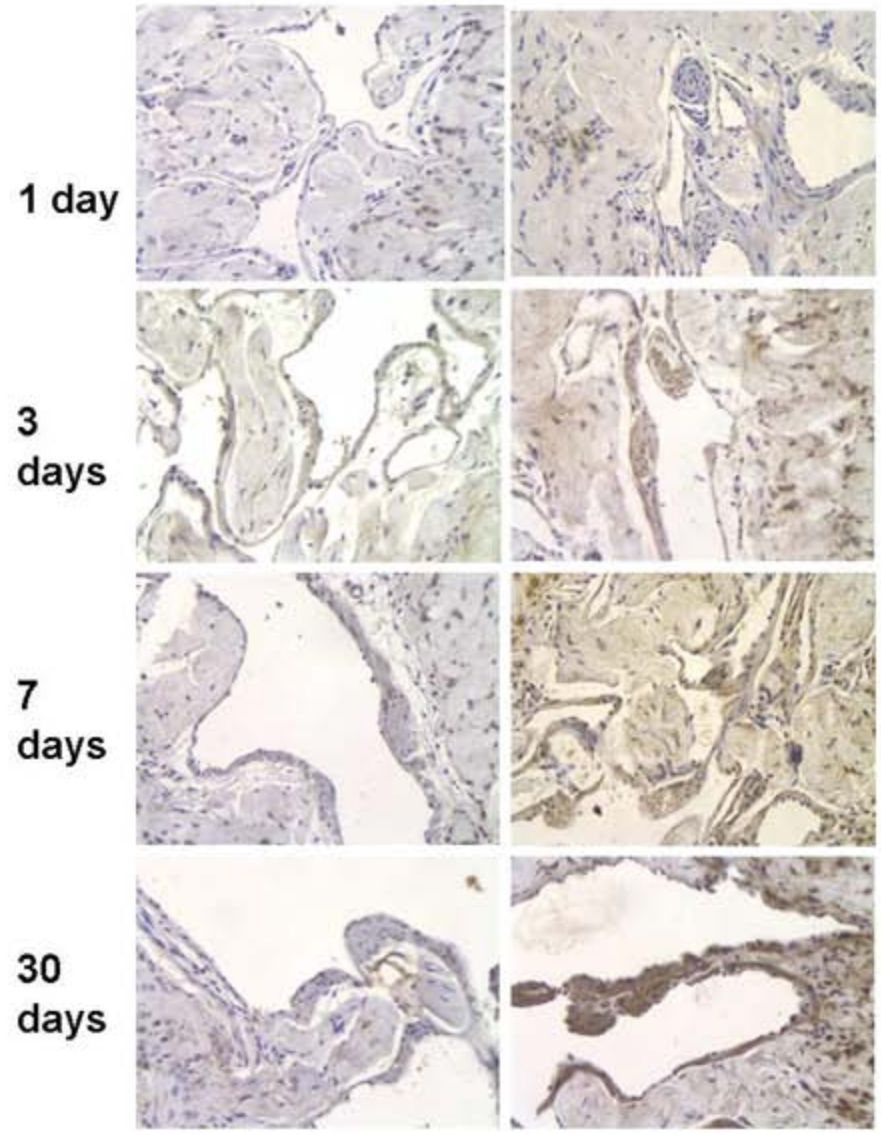

Sham
BCNR

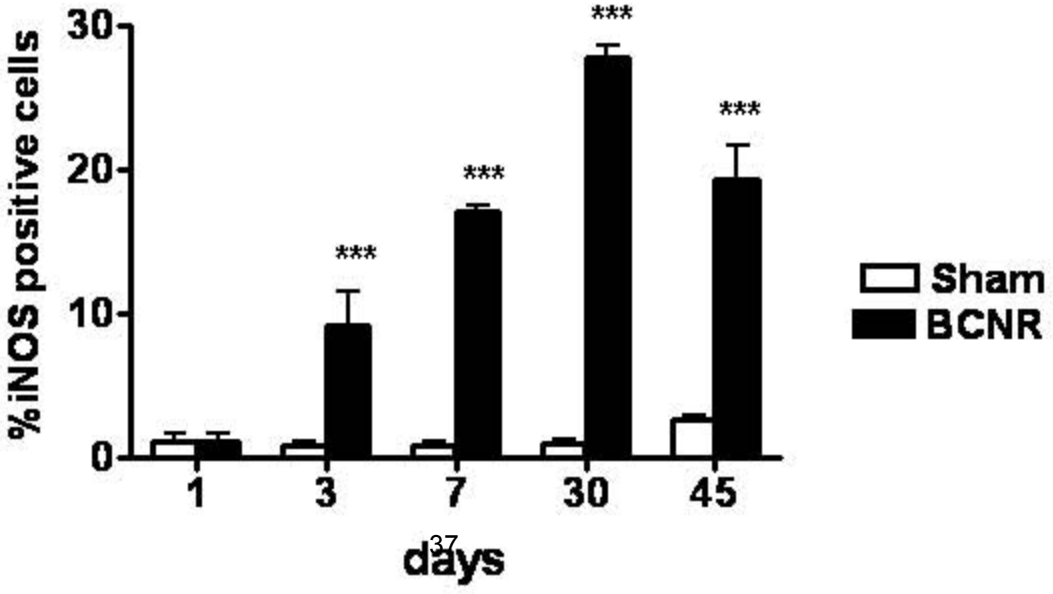

A
SHAM
1 day
3 days
7 days
30 days

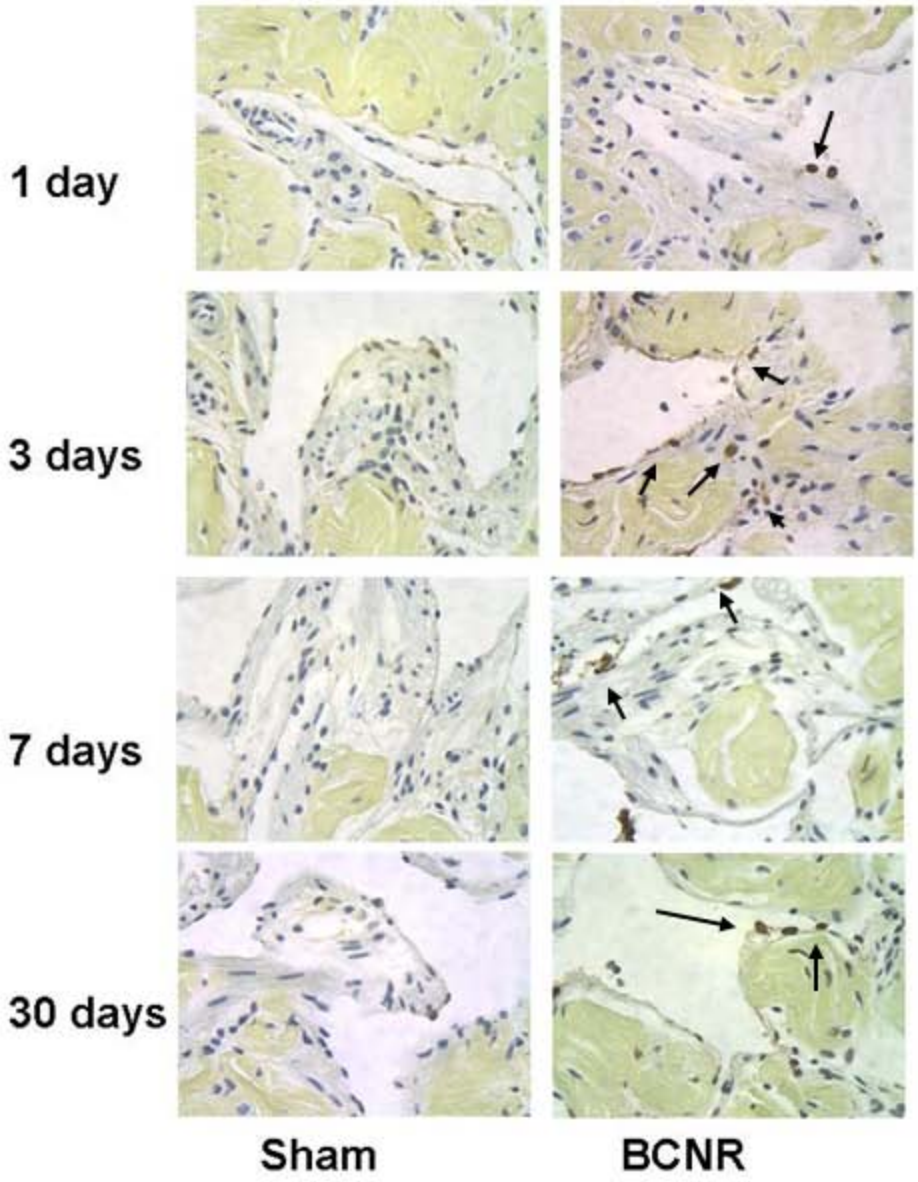

B
SHAM
BCNR
1 day
3 days
7 days
30 days

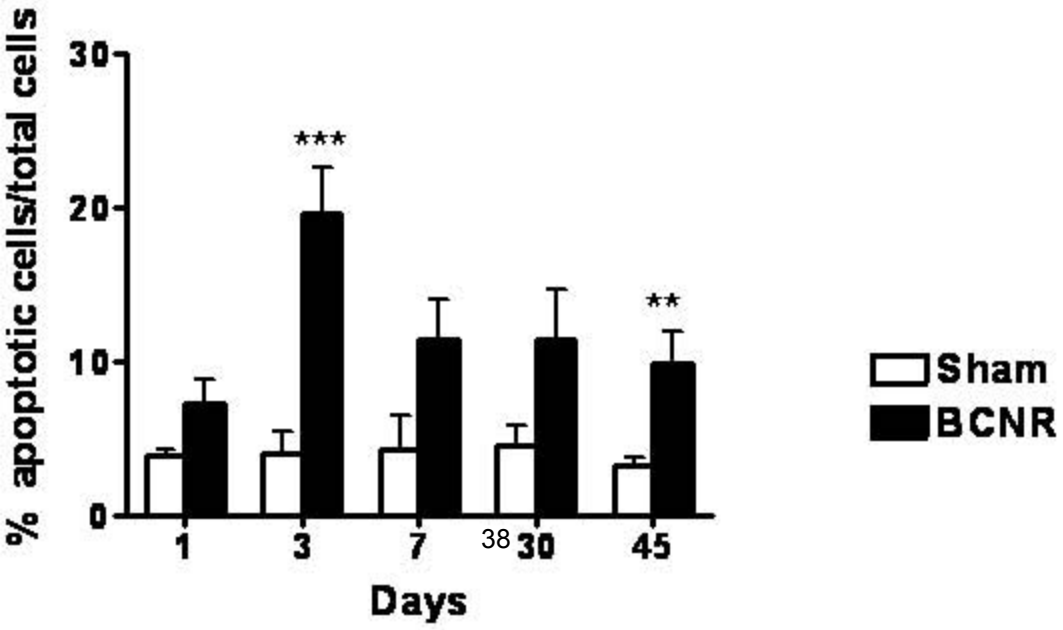

% eNOS area/total area
20
10
0
1
3
7
30
Days

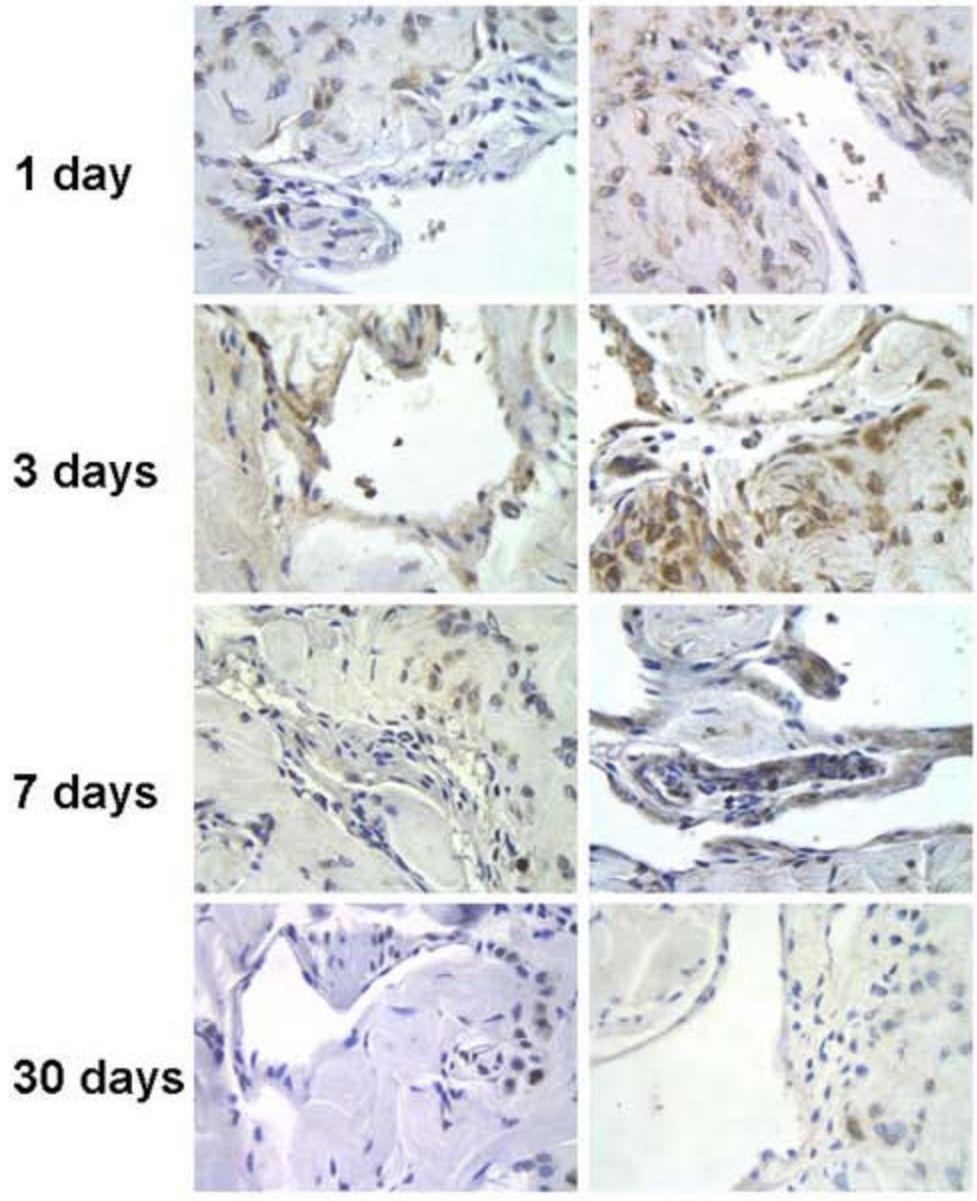

eNOS Optical density
0.25
0.20
0.15
0.10
0.05
0.00
1
3
7
30
Days
Sham
BCNR

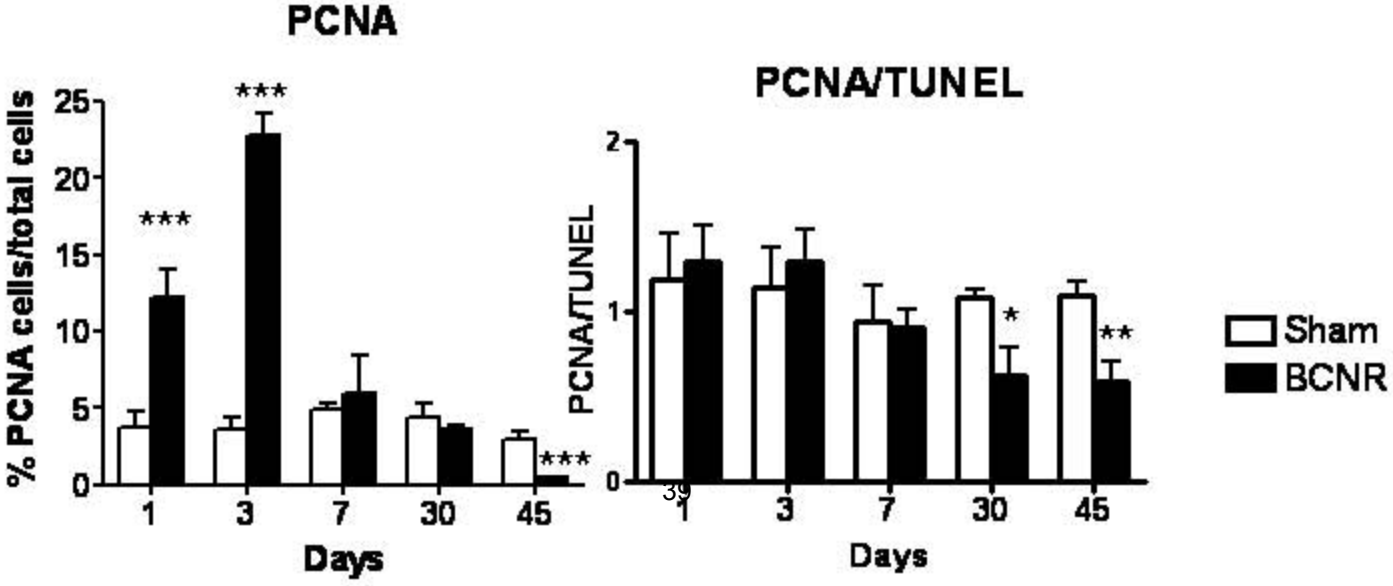

1 day
3 days
7 days
30 days
Sham
BCNR

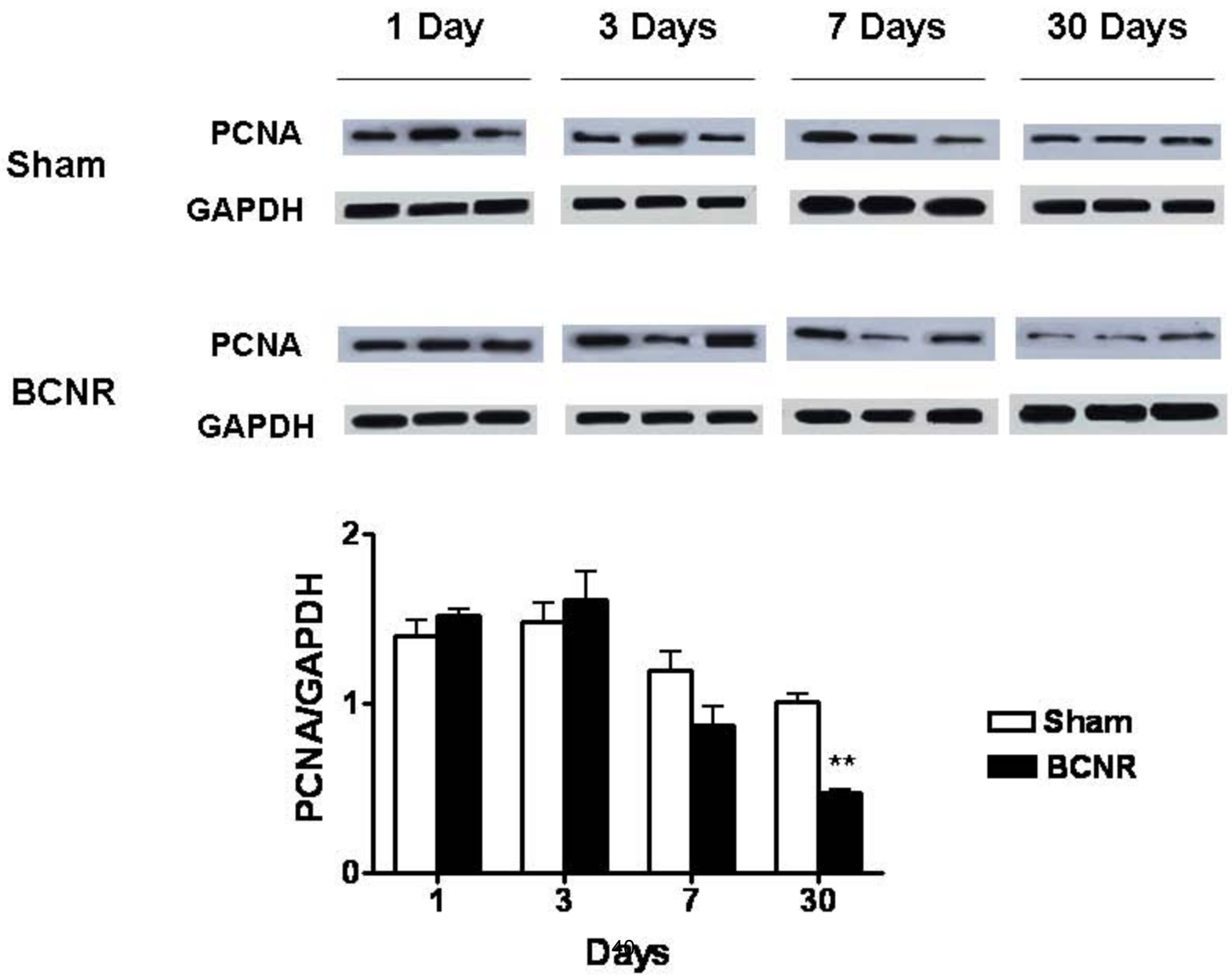

% iNOS positive cells
30
20
10
0
***
***
***
***
1
3
7
30
45
days
Sham
BCNR


1 day
3 days
7 days
30 days
Sham
BCNR


% apoptotic cells/total cells
30
20
10
0
***
**
1
3
7
30
45
Days
Sham
BCNR


1 day
3 days
7 days
30 days
Sham
BCNR


PCNA
% PCNA cells/total cells
25
20
15
10
5
0
***
***
***
1
3
7
30
45
Days
PCNA/TUNEL
PCNA/TUNEL
2
1
0
*
**
1
3
7
30
45
Days
Sham
BCNR


1 Day
3 Days
7 Days
30 Days
Sham
PCNA
GAPDH
BCNR
PCNA
GAPDH
PCNA/GAPDH
2
1
0
1
3
7
30
Days
**
Sham
BCNR

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ORIGINAL ARTICLE

# Long-term continuous sildenafil treatment ameliorates corporal veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats

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**It was recently reported in the rat that vardenafil given in a continuous long-term manner was successful in preventing smooth muscle fibrosis in the penile corpora cavernosa and corporal veno-occlusive dysfunction (CVOD) that occur following bilateral cavernosal nerve resection (BCNR), a model for human erectile dysfunction after radical prostatectomy. To expand on this finding and to determine whether this effect was common to other PDE5 inhibitors, and occurred in part by stimulation of the spontaneous induction of inducible nitric oxide synthase (iNOS, also known as NOS2), male Fischer 344 rats ($N = 10$/group) were subjected to either BCNR or unilateral cavernosal nerve resection (UCNR) and treated with sildenafil (20 mg kg$^{-1}$ day$^{-1}$) in the drinking water daily for 45 days. Additional BCNR groups received L-NIL (6.7 mg kg$^{-1}$ day$^{-1}$) as inhibitor of iNOS activity, with or without concurrent sildenafil administration. It was determined that sildenafil, like vardenafil, (1) prevented the 30% decrease in the smooth muscle cell/collagen ratio, and the 3–4-fold increase in apoptosis and reduction in cell proliferation, and partially counteracted the increase in collagen, seen with both UCNR and BCNR; and (2) normalized the CVOD, measured by dynamic infusion cavernosometry, induced by both BCNR and UCNR. The long-term inhibition of iNOS activity exacerbated corporal fibrosis and CVOD in the BCNR rats, but sildenafil functional effects were not affected by L-NIL. These data suggest that the salutary effects of continuous long-term PDE5 inhibitors on erectile function post-cavernosal nerve resection involve their ability to prevent the alterations in corporal histology induced by cavernosal nerve damage, in a process apparently independent from endogenous iNOS induction.**
*International Journal of Impotence Research* advance online publication, 20 September 2007; doi:10.1038/sj.ijir.3901612

**Keywords:** erectile dysfunction; radical prostatectomy; nerve damage; PDE5 inhibitors; smooth muscle; fibrosis

## Introduction

Erectile dysfunction is a common complication following radical prostatectomy that affects the quality of life of both the patient and his partner. In addition, many men who have been diagnosed with early stage prostate cancer and are candidates for radical prostatectomy avoid this surgery primarily because of the fear of developing this side effect.[1–3] The main cause of erectile dysfunction in this patient population is corporal veno-occlusive dysfunction (CVOD), which occurs when the corporal smooth muscle is unable to relax sufficiently and let the intracorporeal pressure adequately compress the subtunical veins, which prevents the egress of blood out of the corpora during tumescence.[4] Regardless of its cause, when the number of smooth muscle cells (SMC) decreases and/or the collagen content increases, the corporal tissue loses its normal compliance and is prone to developing CVOD.

During a radical prostatectomy, the cavernosal nerves are susceptible to injury. This not only impairs

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the normal nitrergic neurotransmission which initiates the normal erectile response, but can also lead to the loss of SMC and corporal fibrosis.[5–8] It is this alteration in the corporal smooth muscle to collagen ratio that is assumed to lead to CVOD.[9] As a result, both spontaneous erections and the response to vasoactive drugs, including the oral PDE5 inhibitors when given on demand to elicit an erection, can be adversely affected.[3,10]

Several recent studies addressing CVOD in the aged or diabetic rat suggests that this form of erectile dysfunction is associated with the loss of SMC and excessive deposition of collagen fibers within the corpora, and that the long-term continuous administration of PDE5 inhibitors may counteract these processes.[11–13] It is likely that this occurs through the maintenance of high levels of cGMP, since this compound reduces collagen synthesis and the activation of the pro-fibrotic TGFβ1 pathway and protects SMC from apoptosis, while stimulating the spontaneous induction of inducible nitric oxide synthase (iNOS, also known as NOS2).[14–23] The expression of iNOS in certain non-immunological tissues is assumed to be a defense mechanism against fibrosis.[24–29] The nitric oxide produced by iNOS, besides inhibiting collagen synthesis and the TGFβ1 pathway, also quenches reactive oxygen species, and in some cases, the differentiation of fibroblasts to myofibroblasts, the cells that produce collagen in many fibrotic conditions.[30–32]

In a recent study, we have shown that the PDE5 inhibitor, vardenafil, given for 45 days in the drinking water to rats subjected to bilateral cavernosal nerve resection (BCNR)[33–35] prevented the development of CVOD and the underlying SMC loss and fibrosis in the corpora cavernosa.[36] An antifibrotic effect by vardenafil and sildenafil was observed in the penile tunica albuginea in the rat model of Peyronie's disease, and in the case of sildenafil in the aged corpora smooth muscle.[37,38,12] In order to confirm and expand those findings by studying not only BCNR, but also unilateral cavernosal nerve resection (UCNR), and by using another PDE5 inhibitor, we have determined: (a) how a unilateral nerve injury compares to the more severe BCNR, and (b) whether sildenafil has similar anti-fibrotic properties as vardenafil and works via iNOS induction.

## Materials and methods

*Animal treatments*

Five-month-old male Fisher 344 rats (Harlan Sprague–Dawley, San Diego, CA, USA) were treated with an IACUC-approved protocol, and divided as follows ($n = 10$/group): A (sham-operated); B (UCNR), C (UCNR + sildenafil), D (BCNR) and E (BCNR + sildenafil). The drug was given in the drinking water for 45 days (water intake *ad libitum*). Nerve resection was performed as described.[33,35,36] In the sham-operated group, both cavernosal nerves were identified but not resected. In the other groups, the main cavernosal nerves were resected by removing a 3-mm segment uni- or bilaterally. Sildenafil (Pfizer Ltd, Sandwich, UK) was dissolved in the drinking water ($0.3\,mg\,ml^{-1}$), as described previously.[12] The drinking volume was determined daily, and the body weight was recorded weekly. The daily sildenafil dose ($20\,mg\,kg^{-1}$) was approximately equivalent to a single 200-mg tablet daily dose in men, when corrected for differences in total body surface area.[12,38] Treated animals were switched to regular drinking water 1 day prior to cavernosometry, as a washout process. In a subsequent experiment, treatments for groups D and E were repeated, but receiving or not the inhibitor of iNOS activity L-N6-(1-iminoethyl)-lysine (L-NIL) in the drinking water at $100\,mg\,l^{-1}$ (calculated dose:$6.7\,mg\,kg^{-1}\,day^{-1}$) ($n = 8$/group).

*Dynamic infusion cavernosometry*

It was performed as described.[11–13] In brief, the basal intracavernosal pressure (ICP) was recorded, and 2 mg papaverine was administered through a cannula into the corpora cavernosa. The ICP was recorded 5 min later as the 'ICP after papaverine'. After complete detumescence, saline was infused through another cannula, increasing the infusion rate by $0.05\,ml\,min^{-1}$ every 10 s, until the ICP reached 100 mm Hg ('infusion rate'). Then the infusion was adjusted to hold the ICP around 100 mm Hg ('maintenance rate'). The 'drop rate' was determined by recording the decrease in ICP within the next 1 min after the infusion was stopped.

*Histochemistry and immunohistochemistry*

After cavernosometry, the rats were killed and the middle regions of the skin-denuded penile shafts were fixed overnight in 10% formalin, washed and stored in 70% alcohol at 4 °C until processed for paraffin-embedded tissue sectioning (5 μm). Adjacent sections were used for Masson's trichrome staining for collagen (blue) and SM (red); picrosirius red under polarized microscopy for collagen III (green and green-yellow)/I (red and orange) ratios; and immuno-detection with monoclonal antibodies against α-smooth muscle-actin (ASMA) as an SMC marker (Sigma Kit, Sigma Diagnostics, St Louis, MO, USA), proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation (Chemicon, Temecula, CA, USA), and polyclonal antibodies against transforming growth factor β1 (TGFβ1) (1:200; Promega, Madison, WI, USA) and iNOS (1:500; Calbiochem, La Jolla, CA, USA).[12,37] The specificity of the antibodies was validated by western blot.

Sections were then incubated with biotinylated anti-mouse immunoglobulin G (IgG) for ASMA and PCNA or biotinylated anti-rabbit IgG for iNOS and TGFβ1, followed by avidin–biotin complex (Vector Labs, Burlingame, CA, USA) and 3,3′ diaminobenzidine (Sigma Chemical, St Louis, MO, USA) for PCNA and iNOS, or the ASMA Sigma Kit for ASMA and 3-amino-9-ethylcarbazole. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay was performed with the Apoptag peroxidase detection assay (Chemicon). The sections were counterstained with hematoxylin. The negative controls for immunohistochemistry were performed by replacing the first antibody with the IgG isotype. For the negative control for the TUNEL assay, buffer was substituted for the terminal deoxynucleotidyl transferase enzyme. Testicular sections from old animals were used as a positive control for TUNEL.

*Quantitative image analysis*
It was performed by computerized densitometry using the ImagePro Plus, version 5.1, program (Media Cybernetics, Silver Spring, MD, USA) coupled to an Olympus BHS microscope equipped with an Olympus digital camera.[12,13] For Masson staining, $\times 40$ magnification pictures of the penis composed of one half of the corpora cavernosa but excluding the sinusoidal spaces were analyzed for SM (stained in red) and collagen (stained in blue) and expressed as the SM/collagen ratio. An identical approach was used for the collagen III/I ratios. For ASMA and iNOS staining, only the corpora cavernosa was analyzed in a computerized grid and expressed as the percentage of positive area versus total area of the corpora cavernosa. The intensity of immunostaining was determined as the percentage of integrated optical density in the corpora cavernosa. For the TGFβ1, PCNA and TUNEL determinations, the number of positive cells at $\times 400$ was counted, and the results are expressed as the percentage of positive cells/total cells in the corpora cavernosa. In all cases, two fields at $\times 40$, or eight fields at $\times 400$, were analyzed per tissue section, with at least four matched sections per animal and 6–11 animals per group.

*Quantitative western blots*
Penile homogenates of frozen tissue (100 mg) were obtained in T-PER (PIERCE, Rockford, IL, USA) and protease inhibitors (3 μM leupeptin, 1 μM pepstatin A, 1 mM phenyl methyl sulfonyl fluoride), and centrifuged at 10 000 *g* for 5 min. Supernatant protein (30 μg) was run on 7.5 or 10% (ASMA) polyacrylamide gels, and submitted to western blot immunodetection with a monoclonal ASMA IgG (1:1000;Oncogene-Calbiochem, La Jolla, CA, USA), detecting a 43 kDa band. Membranes were incubated with a secondary polyclonal horse anti-mouse IgG linked to horseradish peroxidase (1:2000; BD Transduction Labs, San Diego, CA, USA), and bands were visualized with luminol (Pierce, Rockford, IL, USA).[12,38] A single positive control was run throughout all gels for each antibody to standardize for variations in exposures and staining intensities. Negative controls were performed omitting the primary antibody. Band intensities were determined by densitometry and corrected by the respective intensities for a housekeeping protein, glyceraldehyde phosphate dehydrogenase, upon reprobing.

*Collagen estimation in fresh tissue*
As previously described, the tissue was homogenized in saline, hydrolyzed with 2N NaOH for 30 min at 120 °C, followed by the estimation of hydroxyproline by a modification of the Neumann and Logan's reaction using Chloramine T and Ehrlich's reagent, against a hydroxyproline standard curve and measuring at 550 nm.[11–13,38] Values were expressed as μg of collagen per mg of tissue.

*Statistical analysis*
The values are expressed as the mean ± s.e.m. The normality distribution of the data was established using the Wilk–Shapiro test. Multiple comparisons were analyzed by a single factor analysis of variance, followed by *post hoc* comparisons with the Newman–Keuls test, according to the GraphPad Prism, version 4.1 for windows (GraphPad Software, San Diego CA, USA). Differences were considered significant at $P<0.05$.

## Results

There was no significant difference in body weights between the groups. Sildenafil treatment did not cause priapism, lethargy, aggressiveness or any other noticeable side effect. Rats were subjected to cavernosometry after a 24 h washout period that reduces sildenafil concentrations to baseline.[12,39] Rats undergoing UCNR and BCNR for 45 days had a significant increase in the drop rate as compared to sham-operated animals, which was higher after BCNR than UCNR (Figure 1 bottom), and this was accompanied by a significant reduction of the response to papaverine (top), but only in the BCNR rats. This confirmed our previous observation that CVOD is induced by BCNR, and showed that UCNR induced a moderate CVOD.[14] Continuous oral treatment with sildenafil normalized the drop rate and response to papaverine (top and bottom) in both the UCNR and BCNR animals.

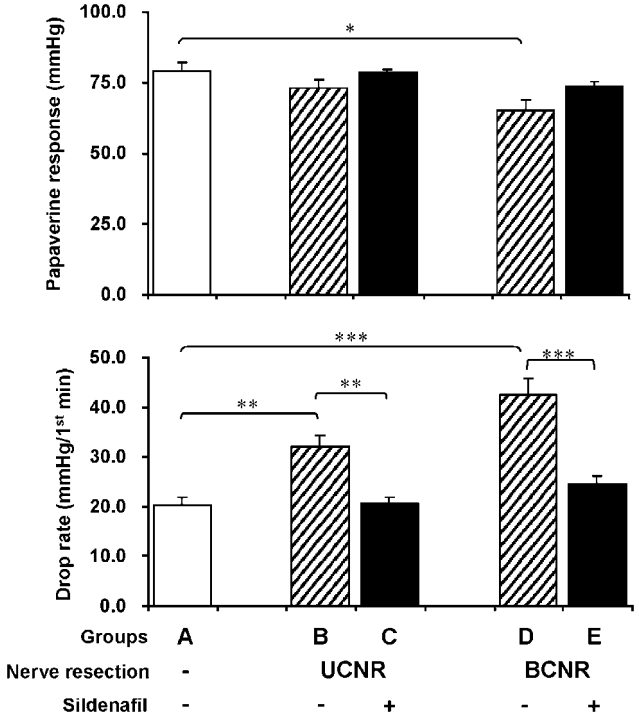


**Figure 1** Effect of unilateral and bilateral cavernosal nerve resection (BCNR) and long-term sildenafil treatment on the erectile function of the rat measured by pharmacological and infusion cavernosometry. Sildenafil treatment was given for 45 days after nerve resection. Top: Response of the intracavernosal pressure to papaverine; Bottom: Response of the intracavernosal pressure to the interruption of saline infusion. A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated BCNR; E: BCNR with sildenafil. $*P<0.05$; $**P<0.01$; $***P<0.001$.

The underlying changes in corporal histology reflected the cavernosometric findings in terms of the smooth muscle/collagen ratio, and SMC content as shown on the representative microphotographs of cross sections of the corpora cavernosa obtained from sham, BCNR untreated and BCNR treated rats, and stained by Masson trichrome or ASMA (Figure 2 top panels). The estimations were performed around the lacunar spaces area where the SMC are concentrated. Both BCNR and UCNR reduced the SMC to collagen ratio, as estimated by Masson trichrome and quantitative image analysis, by about 30% as compared to sham-operated rats, and this was prevented by sildenafil treatment in both the BCNR and UCNR animals (Figure 2 top graph). That these changes in the ratio were due, at least partially, to absolute changes in the SMC content, was shown by quantitative immunohistochemistry for ASMA as a marker of SMC (Figure 2 bottom graph). Both UCNR and BCNR reduced this SMC content by about 60%, at least directly around the lacunar spaces, and sildenafil increased it significantly, but did not fully normalize it.

SMC content was also evaluated in total corpora cavernosa homogenates of the BCNR specimens only, via ASMA immunodetection by quantitative western blot. These preparations of the homogenates include, in addition to corporal SMC, the SMC from the corpus spongiosum and the media of the corporal arteries as well as potential myofibroblasts in the tunica or interstitial connective tissue, whereas the immunohistochemical detection performed above is restricted to corporal SMC. The ASMA band was considerably increased by sildenafil treatment, in comparison to the untreated BCNR rats (Figure 3 top), and the densitometric quantitation (Figure 3 bottom) indicates an over 2-fold increase which agrees with what was observed in the tissue sections.

Collagen content and composition were both affected by nerve resection and by sildenafil treatment. The collagen content was significantly increased by BCNR, but not by UCNR when compared to the sham-operated animals (Figure 4 bottom graph). Continuous long-term sildenafil treatment reduced the collagen content in the BCNR rats, but did not normalize this value. Both BCNR and UCNR decreased the collagen III/I ratio (Figure 4 top graph) as seen previously with vardenafil, although in the UCNR group, this did not reach statistical significance. Sildenafil normalized the collagen III/I ratio in the BCNR rats.[33]

The reduction in SMC was accompanied by a nearly 3-fold increase in apoptosis induced by BCNR and UCNR (Figure 5 top graph), when compared to the sham-operated animals. The % apoptotic index was reduced to normal values in both BCNR and UCNR with continuous long-term sildenafil treatment. The levels of TGFβ1, presumably involved in collagen deposition and SMC loss, were also increased by nearly 2-fold by both UCNR and BCNR (Figure 5 bottom graph), and restored to sham values with continuous long-term sildenafil treatment.

Cell replication within the corpora was evaluated by staining for PCNA (Figure 6 top graph), showing that the % proliferation index was reduced 3–4-fold by UCNR and BCNR compared to sham-operated animals. Continuous long-term sildenafil treatment stimulated cell proliferation restoring the low values seen in the UCNR and BCNR groups back up to the values of the sham controls. As a result, the ratio between the cell proliferation and apoptosis, an indicator of cell turnover, was dramatically reduced by both UCNR and BCNR, and while this was counteracted by sildenafil, normal values were only restored in the UCNR animals.

iNOS, a putative anti-fibrotic and pro-apoptotic factor, was increased 3–4-fold in both the UCNR and BCNR rats when compared to the control animals, as shown by immunohistochemistry (Figure 7). However, in the long-term sildenafil treated animals, which demonstrated a decrease in both fibrosis and apoptosis when compared to the non-sildenafil treated UCNR and BCNR animals, iNOS induction

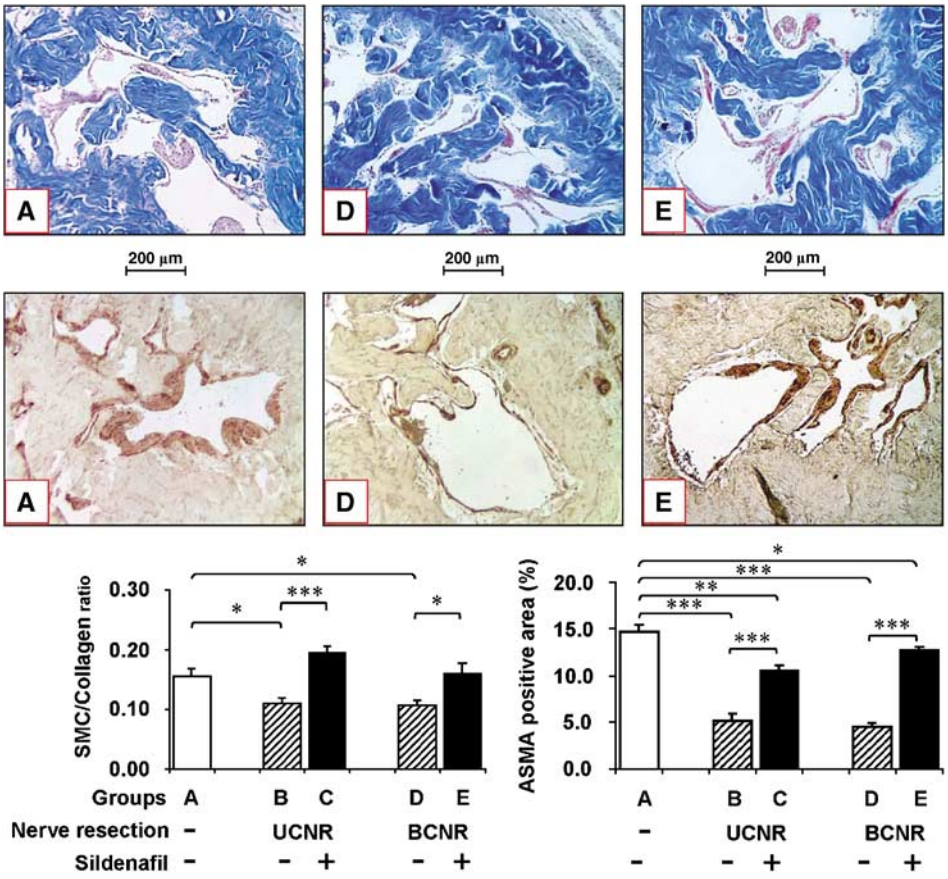


**Figure 2** Effect of unilateral and bilateral cavernosal nerve resection and long-term sildenafil treatment on the smooth muscle/collagen ratio and smooth muscle cell content in the rat corpora cavernosa. Tissue sections from the experimental rat groups in Figure 1 were submitted to Masson's trichrome staining (Top micrographs), and other adjacent sections were immunostained for α-smooth muscle-actin (ASMA) as a smooth muscle cell marker (bottom micrographs). The micrographs depict representative fields ( × 200, bar = 200 μm) and the bar plots show the respective quantitative image analysis. A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated bilateral cavernosal nerve resection (BCNR); E: BCNR with sildenafil. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

was not altered and remained elevated like those of the untreated UCNR and BCNR animals.

To determine whether iNOS spontaneous induction acts as an antifibrotic mechanism subsequent to cavernosal nerve damage, and whether it plays any role in the protection exerted by sildenafil, L-NIL was given to new groups of BCNR rats immediately after the intervention, and these animals were treated or not with sildenafil for 45 days. Other two similar groups did not receive L-NIL. Cavernosometry showed that in the BCNR rats the ICP after papaverine was reduced by L-NIL treatment to the very low value (in mm Hg) of $23.0 \pm 9.7$ as compared to $62.9 \pm 9.8$ in the untreated controls ($P<0.001$). However, L-NIL did not reduce significantly the ICP in the BCNR rats treated with both L-NIL and sildenafil ($65.9 \pm 13.3$) compared with the sildenafil only treated BCNR rats ($69.5 \pm 10.8$).

iNOS long-term inhibition caused also a significant reduction of the SMC/collagen ratio in the sildenafil-untreated BCNR rats, to $0.05 \pm 0.003$ from $0.09 \pm 0.006$ in the control ($P<0.05$), in good agreement to the effects of L-NIL on the erectile response to papaverine. However, despite the lack of significant inhibition on the functional effects of sildenafil, L-NIL moderately interfered with sildenafil effects on the SMC/collagen ratio, by reducing this value from $0.15 \pm 0.01$ to $0.10 \pm 0.006$ ($P<0.001$).

## Discussion

These results confirm and extend our previous work with vardenafil in BCNR rats by showing that UCNR is *per se* deleterious to the corpora tissue, resulting in a reduction in the SMC content, and SMC proliferation, while increasing collagen content, TGFβ1, apoptosis and iNOS induction, to nearly the same extent as BCNR.[33] In addition, the only parameters that UCNR affected significantly less than BCNR were CVOD and collagen deposition thereby suggesting that a certain level of collagen

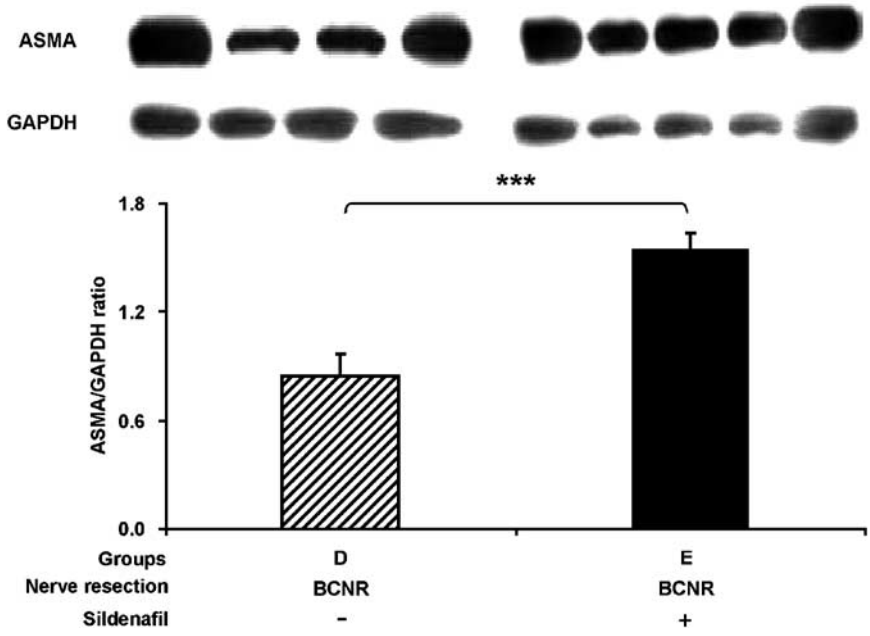


**Figure 3** Effect of long-term sildenafil treatment on the expression of α-smooth muscle-actin (ASMA) in total penile shaft tissue from rats subjected to bilateral cavernosal nerve resection. Homogenates from total penile shaft tissue were submitted to western blot for ASMA. Top: representative pictures of the gels depicting the ASMA band. Bottom: densitometric analysis. $***P<0.001$.

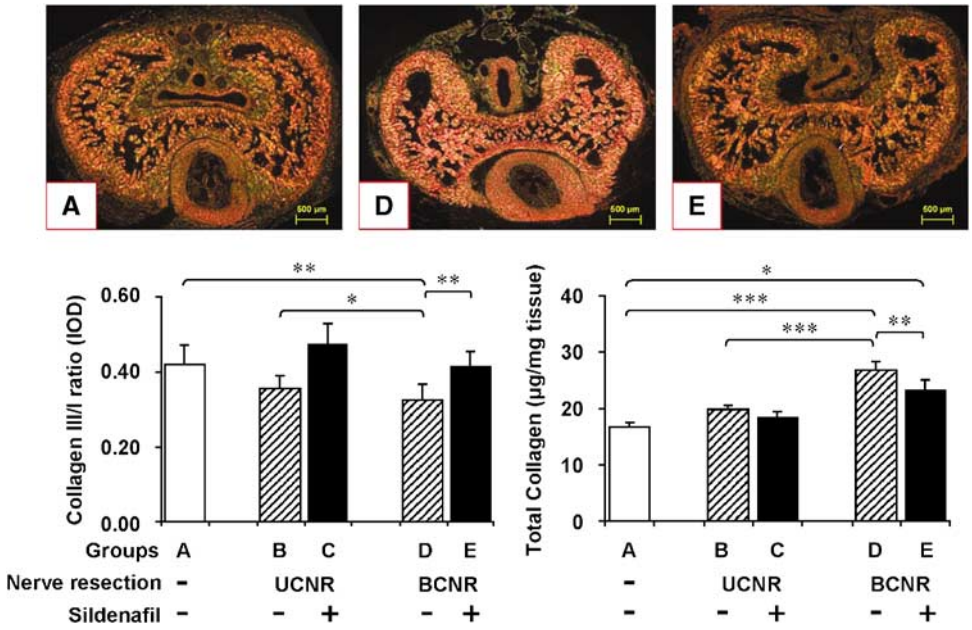


**Figure 4** Effect of unilateral and bilateral cavernosal nerve resection and long-term sildenafil treatment on the collagen III/I ratio and total collagen content in the rat corpora cavernosa. Adjacent tissue sections to those in Figure 2 were submitted to Picro-Sirius red staining and visualized under polarized light (top micrographs). Frozen penile specimens were used to determine the total collagen content by a hydroxyproline assay (bottom). The micrographs depict representative fields taken at $\times 40$ with the method of overlapping fields and assembled to represent the whole penis (bar: 500 µm) to show the distribution of collagen I (red/orange) and collagen III (green–greenish-yellow), and the bar plots show the quantitative image analysis. A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated bilateral cavernosal nerve resection (BCNR); E: BCNR with sildenafil. $*P<0.05$; $**P<0.01$ $***P<0.001$.

deposition may be necessary to cause a frank CVOD. The PCNA results confirmed our previous contention that there is a basal SMC proliferation occurring in the corpora cavernosa, similarly to what occurs in the arterial media, which is reduced by cavernosal nerve damage.[40] Furthermore, oral continuous

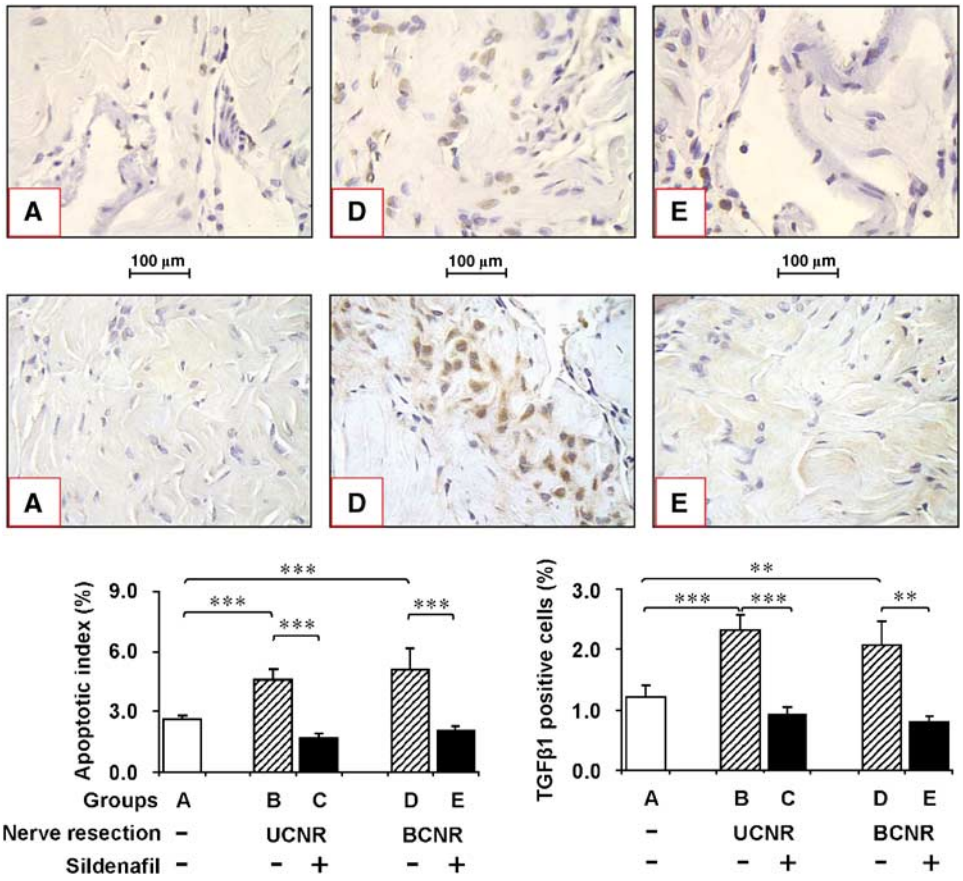


**Figure 5** Effect of unilateral and bilateral cavernosal nerve resection and long-term sildenafil treatment on apoptosis and TGFβ1 expression in the rat corpora cavernosa. Adjacent tissue sections to those in the preceding figures were submitted to TUNEL staining (top micrographs), and other sections were immunostained for TGFβ1 (bottom micrographs). The micrographs depict representative fields ($\times 400$, bar $= 100\,\mu m$) and the bar plots show the quantitative image analysis. A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated bilateral cavernosal nerve resection (BCNR); E: BCNR with sildenafil. $^{**}P<0.01$; $^{***}P<0.001$.

long-term treatment with sildenafil, as opposed to the sporadic on demand treatment that is used to elicit an erection, normalized the physiological and for the most part the tissue composition values in the BCNR rats. And finally, as seen with vardenafil, the continuous long-term treatment with sildenafil did not cause noticeable adverse side effects. Moreover, a prolonged treatment with sildenafil in the rat does not induce tachyphylaxis by PDE5 upregulation.[41]

Because UCNR and BCNR represent from an experimental point of view what may result from either a partial or complete cavernosal nerve injury, respectively, during a radical prostatectomy, these observations together with those previously shown with vardenafil support the continuous use of oral PDE5 inhibitors post-prostatectomy for preventing the histological changes in the corpora that CVOD and resultant erectile dysfunction. The dose that we have used in this present study is likely to be considered excessive for routine clinical use, since it is roughly equivalent to 200 mg day$^{-1}$ for men.[12,38] However, we chose this dose based on a parallel study in the aged rat where sildenafil was given in a similar continuous and long-term manner to prevent the histological and physiological changes associated with aging related erectile dysfunction which has been shown to be primarily due to CVOD.[12]

This 20 mg kg$^{-1}$ day$^{-1}$ dose of sildenafil in the rat is about 10-fold higher than the one we used for vardenafil in our BCNR rat model and is within the dosages that have been tested in continuous administration in experimental animals and even in patients for conditions such as pulmonary hypertension.[33,42–44] With the exception of the anti-apoptotic effect that we found with sildenafil in the current study which we did not see with vardenafil in our previous report, both drugs elicited the same effects despite the difference in dosage.[33] Therefore, it is likely that a lower dose of sildenafil would also be as effective. Obviously, a dose response study should be explored in this experimental nerve resection model using retrolingual daily doses, although the pharmacokinetics of sildenafil clearance may require a higher dose.[37,39]

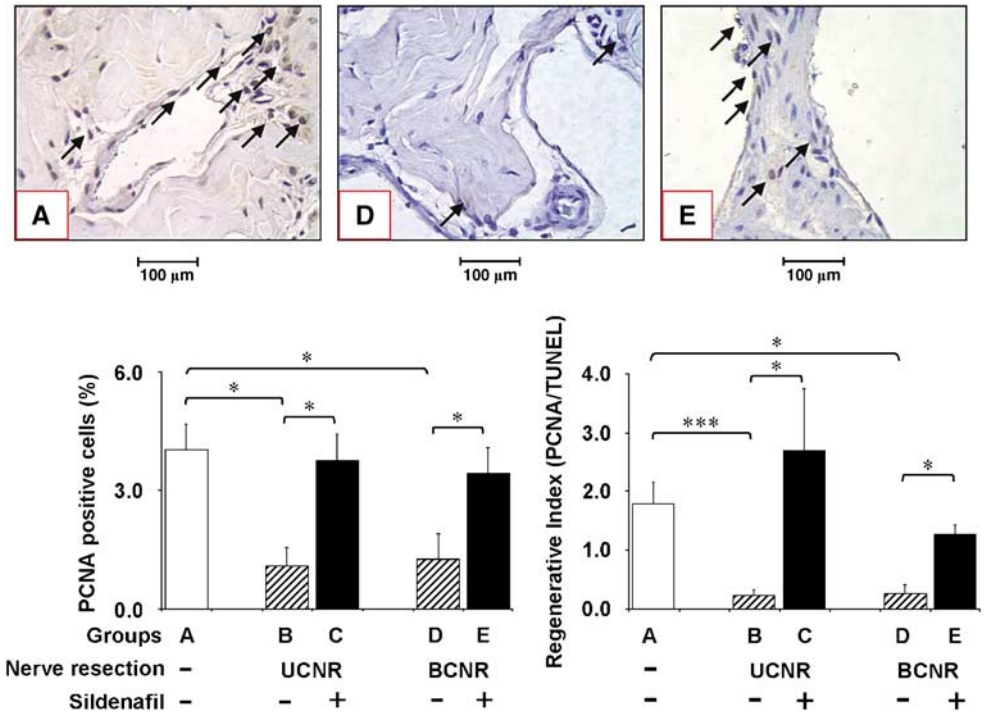


**Figure 6** Effect of unilateral and bilateral cavernosal nerve resection and long-term sildenafil treatment on cell proliferation and turnover in the rat corpora cavernosa. Adjacent tissue sections to those in the preceding figures were submitted to PCNA immuno-staining (top micrographs), and the bar plots show the quantitative image analysis. The micrographs depict representative fields ( × 400, bar = 100 μm). The ratio between the proliferation index and the apoptotic index calculated from Figure 4 was plotted (bottom) A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated bilateral cavernosal nerve resection (BCNR); E: BCNR with sildenafil. $*P<0.05$; $***P<0.001$.

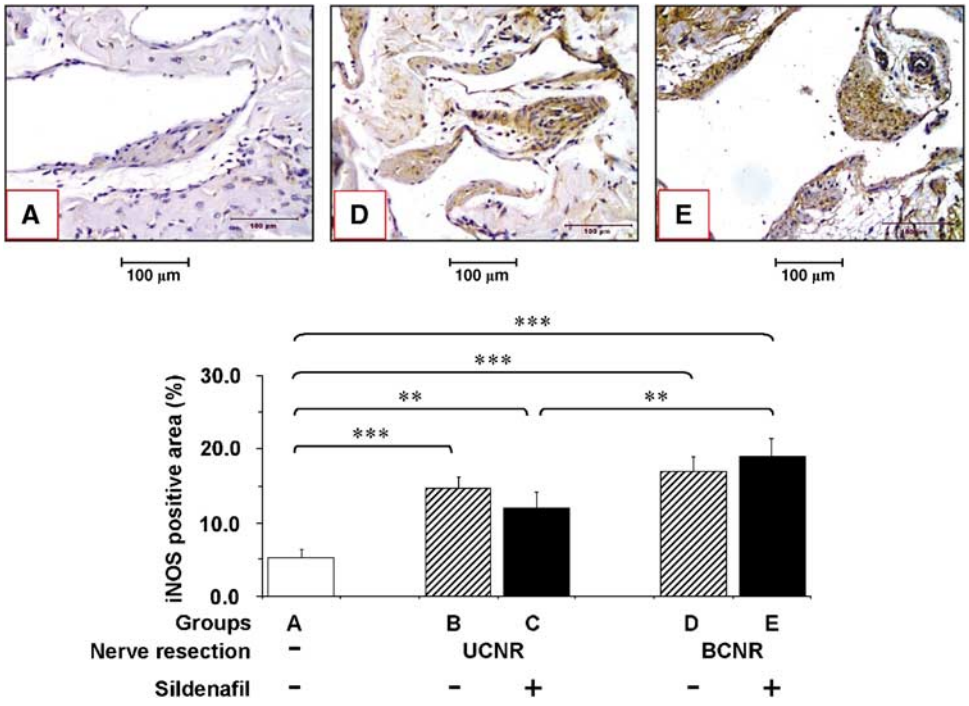


**Figure 7** Effect of unilateral and bilateral cavernosal nerve resection and long-term sildenafil treatment on inducible nitric oxide synthase (iNOS) expression in the rat corpora cavernosa. Adjacent tissue sections to those in the preceding figures were submitted to iNOS immuno-staining. The micrographs depict representative fields ( × 400, bar = 100 μm) and the bar plots show the quantitative image analysis. A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated bilateral cavernosal nerve resection (BCNR); E: BCNR with sildenafil. $**P<0.01$; $***P<0.001$.

Based on these current findings in the UCNR and BCNR models with sildenafil and those previously with vardenafil in the BCNR model, together with that observed in the aged rat treated with sildenafil, we believe that the PDE5 inhibitors possess antifibrotic activity which in the penile tissue may lead to the prevention or delay in the onset or progression of CVOD regardless of its etiology.[33,12] Although cGMP was not estimated in the penile tissue in this study because the rats were killed after a 24 h washout, we assume that the high cGMP levels generated by PDE5 inhibitors are what counteracts fibrosis and protects the corporal smooth muscle.[12,33,37,38] This assumption is based on the known inhibitory effects of cGMP on collagen synthesis and the TGFβ1 pathway, and its vasculoprotective effects on arterial SMC.[14–17,20,45–47] However, the anti-apoptotic and pro-proliferative effects of sildenafil that we found in the corporal smooth muscle do not agree with the fact that cGMP inhibits vascular SMC proliferation.[48,49] Since the effects of BCNR on the corporal histology and pharmacokinetics are the same as those seen in the aged rat, we assume that cGMP effects on corporal SMC may be modulated by some specific features in the corporal SMC themselves or the tissue milieu, that are not operative in the vascular SMC.[12]

Another still unresolved question is the role of iNOS induction in corporal atrophy after cavernosal nerve damage. Studies on liver and kidney fibrosis in the iNOS knock-out mouse, and our previous work in Peyronie's disease and its animal models, vaginal fibrosis, aging-related arterial media fibrosis, and in corporal fibrosis, suggests that despite iNOS may be initially induced during an early inflammatory process, its main role is as an antifibrotic agent.[12,13,24–29,32,33,37,38,50] This may occur via cGMP produced by the nitric oxide from iNOS, and/or the nitric oxide can directly reduce collagen synthesis, myofibroblast formation in the interstitial connective tissue, and reactive oxygen species.[26,30–32] In contrast to our study with vardenafil in the BCNR rat, the slight stimulation of iNOS induction by sildenafil in the BCNR animals was not significant.

It is noteworthy that the high iNOS levels in the presence of sildenafil did not counteract the normalization of the apoptotic index or the upregulation of cell replication exerted by the drug. Since nitric oxide in the vasculature is usually pro-apoptotic and antiproliferative, this would support our assumption regarding the opposite response of corporal and arterial SMC toward the nitric oxide/cGMP pathway, in this respect. The experiment using long-term continuous L-NIL as an iNOS inhibitor supported the view that iNOS acts as a true endogenous antifibrotic agent in the BCNR model, as it was in the aged rat.[29] However, the protective effects of long-term continuous sildenafil on restoring erectile function and reducing corporal fibrosis in this model do not seem be mainly mediated by iNOS induction, since L-NIL did not significantly affect the functional response to sildenafil, and interfered only partially with the improvement of the SMC/collagen ratio exerted by the PDE5 inhibitor.

We acknowledge that since this work, as well as the preceding one with vardenafil, has focused on SMC number and fibrosis, we cannot exclude that sildenafil action may also involve protecting the SMC relaxation/contractile phenotype and/or the integrity of the cavernosal endothelium, but we believe the intracellular/extracellular composition of the corporal smooth muscle is the main target of this drug when given long-term, since this balance is responsible for tissue compliance to relaxation by nitric oxide.[33,51–55]

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# Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction that occurs after cavernosal nerve resection

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## OBJECTIVES

To determine whether a long-term single daily oral dose of a longer half-life phosphodiesterase-5 (PDE5) inhibitor, tadalafil, has a similar effect to that of the shorter half-life PDE5 inhibitors sildenafil and vardenafil, and can prevent the fibrosis and resultant corporal veno-occlusive dysfunction (CVOD) occurring after cavernosal nerve (CN) injury.

## MATERIALS AND METHODS

Male rats (10 per group) had either a sham operation, unilateral CN resection (CNR) or bilateral CNR, and were left untreated or given retrolingually 5 mg/kg per day of tadalafil. After 45 days, CVOD was assessed via cavernosometry, and the underlying corporal tissue changes were examined by immunohistochemistry and histochemistry (followed by quantitative image analysis), Western blots, and ad hoc methods.

## RESULTS

Tadalafil treatment normalized the low response to papaverine and high drop rate in the intracavernosal pressure measured by cavernosometry after CNR compared with sham-operated rats. Tadalafil also normalized the increase in penile shaft collagen content, and the reduction in corporal smooth muscle cell (SMC) content, SMC/collagen, and replication index, and improved the lower collagen III/I ratio and the increase in apoptotic index, caused by CNR, compared with sham operation. There were no effects of tadalafil on increased transforming growth factor β1, inducible nitric oxide synthase and xanthine oxidoreductase levels.

## CONCLUSIONS

A long-term single daily dose of tadalafil prevented CVOD and the underlying corporal fibrosis in the rat caused by CN damage, as effectively as the previously reported continuous treatment with vardenafil or sildenafil, through a cGMP-related mechanism that appears to be independent of inducible nitric oxide synthase induction.

## KEYWORDS

erectile dysfunction, nerve-sparing radical prostatectomy, PDE5 inhibitors, inducible nitric oxide synthase, fibrosis, smooth muscle

## INTRODUCTION

Radical prostatectomy (RP) is considered by many to be curative for patients with early-stage prostate cancer. However, because of the potential risk of damage to the cavernosal nerves (CNs) and the subsequent development of erectile dysfunction that affects 60–90% of patients at 1 year after RP [1–4], this surgical option is often declined. In a large study, there was recovery of potency after 5 years in only 28% of cases [2]. Although nerve-sparing (NS) techniques have been developed that attempt to reduce the incidence of erectile dysfunction after RP, its success is not guaranteed and depends on the surgical centre, age of patients, and other factors, being 31–86% of cases in bilateral NS retropubic RP (NSRRP), to 13–56% in unilateral NSRRP [1,3].

Most of the potency rates cited above correspond to patients treated with oral phosphodiesterase-5 (PDE5) inhibitors given on demand to elicit an erection, with 35–75% response rates for sildenafil given after NSRRP, vs 0–15% for non-NSRRP [5,6]. Other reports give responses of 40–50% with vardenafil or tadalafil, after bilateral NSRRP, although these have not been direct comparisons, and patient selection criteria varied [7]. Most of these men, and those who do not respond to the PDE5 inhibitors, have vasculogenic erectile dysfunction when evaluated by duplex ultrasonography and/or dynamic infusion cavernosometry. In one such study, 59% of men showed arterial insufficiency after bilateral NSRRP and a substantial fraction (26%) had 'venous leakage' or corporal veno-occlusive dysfunction (CVOD) [8]. The latter group had the worst prognosis for the return of erectile function 1 year after RP (only 9%).

The clinical evidence suggests that in addition to the CN damage that can occur as a result of RP, the surgically elicited neuropraxia can also lead to alterations within the corpora cavernosa, specifically loss of smooth muscle (SM) and excessive collagen deposition, as

well as a putative endothelial damage to the sinusoids [9–11]. Indeed, experimental studies conducted in the rat showed that CN damage elicited by either resection, freezing or crushing, is accompanied by a complete or partial reduction in the erectile response to electrical field stimulation of the CN and by profound histological changes within the corpora [12–16], similar to that in the human corpora after RP. These alterations consist of a loss of corporal SM cells (SMC) by apoptosis, and an increase in collagen deposition within the corpora, e.g. tissue fibrosis, and this is presumed to be the cause of CVOD in patients after RP.

We have shown in the rat that CN resection (CNR) leads to a spontaneous induction of the inducible nitric oxide synthase (iNOS, also known as NOS2) within the cavernosal SMC [15,16], and proposed that it acts as an antifibrotic compound that attempts to protect the corpora cavernosal histology in the same way that it acts on other tissues undergoing fibrosis, e.g. the penile tunica albuginea, the vagina, or the peripheral arteries [17–22]. iNOS expression presumably produces a steady increase in local NO, that also raises local cGMP levels, and these two products inhibit collagen production and preserve the SM. In the case of NO, it quenches the pro-fibrotic reactive oxygen species generated during oxidative stress.

In this CNR rat model, when the short-acting PDE5 inhibitors, sildenafil or vardenafil, were used long-term and continuously, rather than 'on-demand' as used clinically to induce an erection in men, the histology of the corporal tissue and the dynamic infusion cavernosometric responses in these rats became normal, presumably by increasing local cGMP levels [15,16]. This agrees with and might explain the results obtained by Padma-Nathan [7], giving nightly sildenafil for 9 months to patients with bilateral NSRRP, where after a 4-week discontinuation of treatment, 27% of patients had a return of spontaneous erections.

In the present study, we aimed to determine whether the long-acting PDE5 inhibitor, tadalafil [23,24], given in daily single doses orally, rather than continuously as in the previous studies [15,16], was also effective in preserving the integrity of the corporal histology and the erectile response of rats treated with unilateral (U) or bilateral (B)CNR.

## MATERIALS AND METHODS

Fisher 344 male rats (5 months old; Harlan Sprague-Dawley, San Diego, CA, USA) were treated with an institutionally approved protocol, and randomly divided into the following five groups (10/group): A (sham-operated); B (UCNR), C (UCNR + tadalafil), D (BCNR), and E (BCNR + tadalafil); the CNR was done as described previously [15,16]. In group A, both CNs were identified but not resected. In groups D and E, both CNs and ancillary branches were resected by removing a 3-mm segment, whereas in the respective UCNR groups (B and C) only one of these nerves was resected. Tadalafil (Lilly ICOS, San Francisco, CA, USA), was dissolved in 10% glucose/1% Tween 80 and administered retrolingually once per day, as described previously for vardenafil in another model [25]. The daily tadalafil dose given to these rats (5 mg/kg/day) was about equivalent to a single 50 mg daily dose in men, when corrected for differences in total body surface area [15,16,26]. Treated rats had their tadalafil suspended 3 days before cavernosometry and death, as a 'washout'.

As previously described [15,17,27], the basal intracavernosal pressure (ICP) was recorded, and 2 mg papaverine was administered through a cannula into the corpora cavernosa. The ICP was recorded 5 min later as the 'ICP after papaverine'. After complete detumescence, saline was then infused through another cannula, increasing the infusion rate by 0.05 mL/min every 10 s, until the ICP reached 100 mmHg ('infusion rate'). Then the infusion was adjusted to hold the ICP at ≈100 mmHg ('maintenance rate'). The rate of decrease ('drop rate') was determined by recording the decrease in ICP within the next 1 min after the infusion was stopped.

After cavernosometry, the rats were killed and the middle regions of the skin-denuded penile shafts were fixed overnight in 10% formalin, washed, and stored in 70% alcohol at 4 °C until processed for paraffin-embedded tissue sectioning (5 μm). Adjacent sections were used for Masson's trichrome staining for collagen (blue) and smooth muscle (red); picro-sirius red under polarized microscopy for collagen III (green and green-yellow) and I (red and orange) ratios; and immunodetection with monoclonal antibodies against α-SM actin (ASMA) as a SMC marker (Sigma Diagnostics, St. Louis, MO, USA), proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation (Chemicon, Temecula, CA, USA), and polyclonal antibodies against TGFβ1 (Promega, Madison, WI, USA), and iNOS (Calbiochem, La Jolla, CA, USA), and the oxidative stress marker xanthine oxidoreductase [15–21,28]. The specificity of the antibodies was validated by Western blot.

Sections were then incubated with biotinylated antimouse IgG for ASMA and PCNA or biotinylated antirabbit IgG for iNOS and TGFβ1, followed by avidin-biotin complex (Vector Laboratories, Temecula, CA, USA) and 3,3′diaminobenzidine (Sigma) for PCNA and iNOS, or the ASMA Sigma Kit for ASMA and 3-amino-9-ethylcarbazole. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) assay was used with the Apoptag peroxidase detection assay (Chemicon) [15–21]. The sections were counterstained with haematoxylin. For the negative controls for immunohistochemistry the first antibody was replaced with the IgG isotype. For the negative control for the TUNEL assay, buffer was substituted for the terminal deoxynucleotidyl transferase enzyme. Testicular sections from old rats were used as a positive control for TUNEL.

For quantitative image analysis we used computerized densitometry (ImagePro Plus, version 5.1, Media Cybernetics, Silver Spring, MD, USA) coupled to a microscope equipped with a digital camera [15–21]. For Masson staining, ×100 views of the penis, composed of one half of the corpora cavernosa but excluding the sinusoidal spaces, were analysed for SM (stained red) and collagen (stained blue) and expressed as the SM/collagen ratio. An identical approach was used for the collagen III/I ratios. For ASMA, xanthine oxidoreductase and iNOS staining, only the corpora cavernosa was analysed in a computerized grid, and expressed as the percentage of positive area vs the total area of the corpora cavernosa. The intensity of immunostaining was determined as the percentage of integrated optical density in the corpora cavernosa. For the TGFβ1, PCNA and TUNEL determinations, the number of positive cells at ×200 was counted, and the results expressed as the percentage of positive cells/total cells in the corpora cavernosa. In all cases, four fields at ×100, or eight fields at ×200, were analysed per tissue section, with at least four matched sections per rat and 6–10 rats per group.

*FIG. 1. Effect of long-term treatment with a single daily dose of tadalafil on the erectile function of the rat measured by pharmacological and dynamic infusion cavernosometry. Tadalafil treatment was given daily for 45 days, with a 3-day previous washout. Top, response of the ICP to papaverine. Bottom, response of the ICP to the interruption of saline infusion. Groups are as defined in the Methods.* P **< 0.05; **< 0.01; ***< 0.001.*

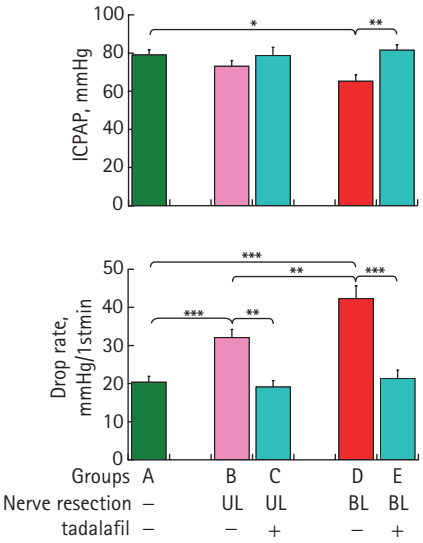
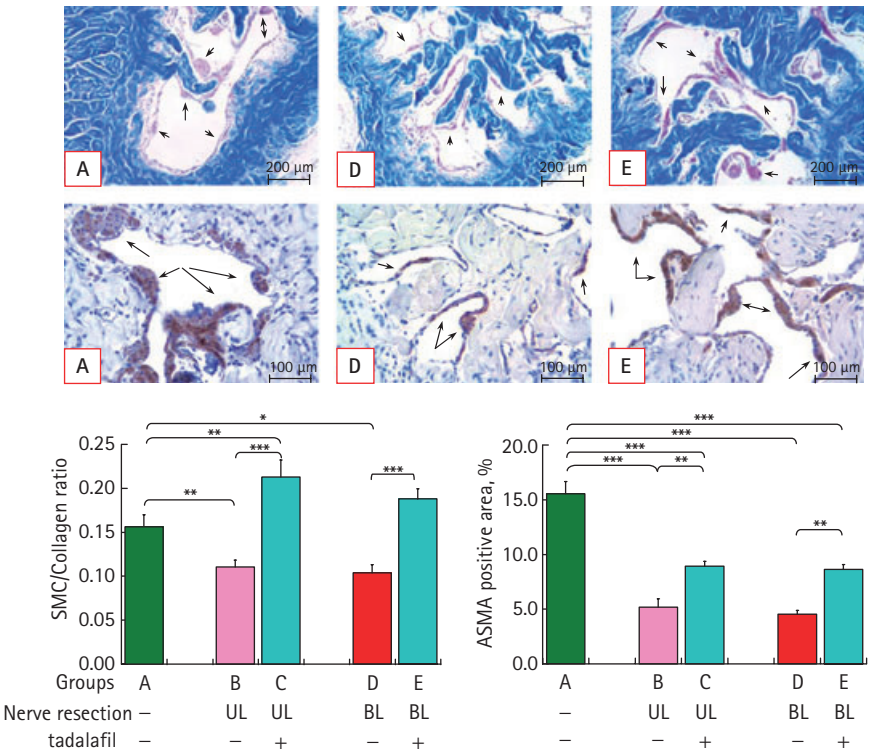



*FIG. 2. Effect of long-term treatment with a single daily dose of tadalafil on the SM/collagen ratio and SMC content in the rat corpora cavernosa. Top panels: Tissue sections from the groups as shown in Fig. 1, were stained with Masson's trichrome: collagen blue, SMC red (arrows; ×100, bar = 200 μm). Bottom panels: Other adjacent sections were immunostained for ASMA as a SMC marker (arrows; ×200, bar = 100 μm). The micrographs depict representative fields and the bar plots show the quantitative image analysis.* P, **< 0.05; **< 0.01; ***< 0.001.*




Penile homogenates of frozen tissue (100 mg) were obtained in a 1% SDS buffer and protease inhibitors (3 μM leupeptin, 1 μM pepstatin A, 1 mM phenyl methyl sulphonyl fluoride), and centrifuged at 10 000 ***g*** for 5 min [16–21,26]. Supernatant protein (30 μg) was run on 7.5% or 10% (ASMA) polyacrylamide gels, and submitted to Western blot immunodetection with a monoclonal ASMA IgG (1 : 1000; Oncogene-Calbiochem), detecting a 43-kDa band. Membranes were incubated with a secondary polyclonal horse antimouse IgG linked to horseradish peroxidase (1 : 2000; BD Transduction Laboratories), and bands were visualized with luminol (Pierce, Rockford, IL, USA) [16–21,26]. A single positive control was run throughout all gels for each antibody to standardise for variations in exposures and staining intensities. For negative controls the primary antibody was omitted. Band intensities were determined by densitometry and corrected by the respective intensities for a housekeeping protein, glyceraldehyde phosphate dehydrogenase (GAPDH), upon reprobing.

For collagen estimation in fresh tissue, as previously described, the tissue was homogenized in saline, hydrolysed with 2 M NaOH for 30 min at 120 °C, followed by the estimation of hydroxyproline by a modification of the Neumann and Logan's reaction using Chloramine T and Ehrlich's reagent, against a hydroxyproline standard curve and measuring at 550 nm [16,21,26]. Values were expressed as μg of collagen per mg of tissue.

The values are expressed as the mean (SEM); the normality distribution of the data was established using the Wilks-Shapiro test. Multiple comparisons were analysed by a single-factor ANOVA, followed by post hoc comparisons with the Newman-Keuls test, with differences considered significant at $P < 0.05$.

## RESULTS

CNR lead to moderate CVOD and underlying corporal fibrosis that was prevented by long-term daily treatment with tadalafil. No side-effects, e.g. lethargy, priapism, aggressiveness, or hyperactivity were noted in the rats treated with tadalafil. Compared with group A, the peak ICP after papaverine administration was significantly reduced by BCNR (Fig. 1, top D vs A), but remained virtually unchanged by UCNR (top B vs A). Tadalafil administered beginning on day 1 after surgery restored, in the BCNR rats, the normal ICP seen in group A (E vs A). On saline infusion into the detumescent penis, the drop rates were very low in group A (bottom, Fig. 1A), confirming normal CVO. However, in the UCNR rats, the drop rate was 1.5 times higher than in group A (B vs A), suggesting a moderate CVOD, and this was increased to more than twice the difference after BCNR (D vs A). In both UCNR and BCNR, tadalafil restored the normal drop rates (C and E vs A).

There was a lower relative area occupied by SMC vs collagen (SMC/collagen ratio) in the corpora cavernosa on with Masson trichrome staining in the BCNR group than in group A (Fig. 2 top, D vs A), and this reduction was

*FIG. 3. Effect of long-term treatment with a single daily dose of tadalafil on the expression of ASMA in total penile shaft tissue from rats after BCNR. Homogenates from total penile shaft tissue were assessed by Western blot for ASMA. Top: representative pictures of the gels depicting the ASMA band, and the housekeeping GAPDH band. Bottom: densitometry analysis. **P < 0.01.*

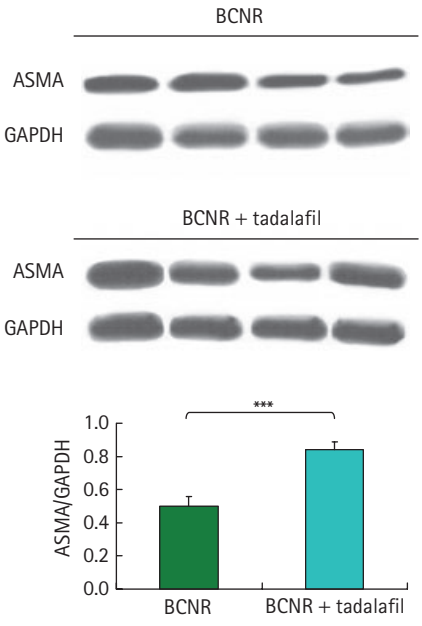



*FIG. 4. Effect of long-term treatment with a single daily dose of tadalafil on the total collagen content and the collagen III/I ratio in the rat corpora cavernosa. Frozen penile specimens were used to determine the total collagen content by a hydroxyproline assay (top bar graph). Adjacent tissue sections to those in Fig. 2 were stained with picro-sirius red and visualized under polarized light (bottom bar graph). The bar plots show the quantitative image analysis. Groups and P values as in Fig. 1.*

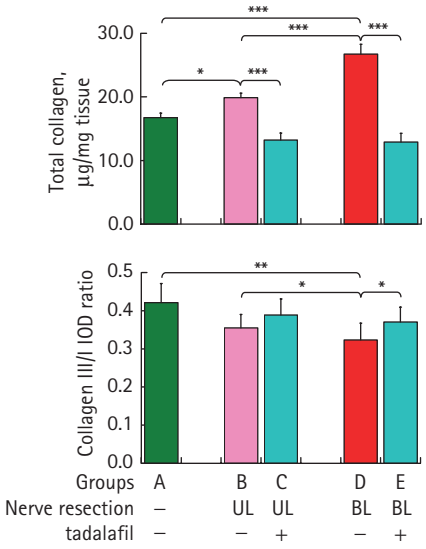
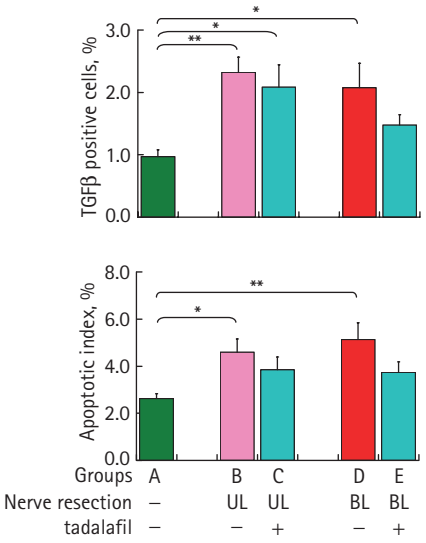



*FIG. 5. Effect of long-term treatment with a single daily dose of tadalafil on apoptosis and TGFβ1 expression in rat corpora cavernosa. Adjacent tissue sections to those in the preceding figures were immunostained for TGFβ1, and other sections assessed by TUNEL staining. The bar plots show the quantitative image analysis. Groups and P values as in Fig. 1.*




apparently counteracted by tadalafil (E vs A). In both the UCNR and BCNR groups, image analysis showed that the decrease in SMC/collagen was moderate (35–40%) (bottom, B and D vs A), but tadalafil (C and E) virtually normalized this ratio. The changes in the SMC/collagen ratio induced by BCNR were due mostly to a considerable decrease in the SMC compartment, as shown by immunodetection for ASMA in adjacent tissue sections, which was partly prevented by tadalafil (Fig. 3 top D and E, vs A). Image analysis confirmed the visual inspection, with an ≈70% decrease in ASMA staining in both UCNR and BCNR, a change that was reduced to only 39% in both groups on tadalafil treatment (Fig. 3, middle). To corroborate the effect of tadalafil on the SMC, the expression of ASMA was estimated by Western blot in total penile shaft homogenates that contained SM not only from the corpora cavernosa but also from the corpus spongiosum and the media of the penile arteries. In the BCNR rats, Fig. 3 (bottom) shows a representative view of the 42-kDa band for ASMA in some of the specimens from the tadalafil-treated (E) vs untreated (D) rats, indicating that tadalafil increased the ASMA content by 60%.

The total content of collagen was evaluated in penile shaft tissue hydrolysates by the hydroxyproline assay (Fig. 4). UCNR (B) and BCNR (D) increased collagen by 19% and 59%, respectively, compared with group A, and tadalafil reduced these values even below (C and E) the normal content. However, there were no significant changes in the collagen III/I ratio with CNR or tadalafil treatment, as evaluated by the picro-sirius red staining/polarized light visualization of the corpora, where collagen III is seen in green/greenish yellow and collagen I in orange/yellowish (not shown).

The loss of corporal SM induced by CNR was associated with an increase in apoptosis and a reduction in cell replication, and this was counteracted by long-term daily tadalafil treatment.

The effects of tadalafil on corporal SMC apoptosis were assessed with the TUNEL assay, as depicted in Fig. 5, which shows greater programmed cell death after BCNR than in group A, and this in turn was reduced by tadalafil (top, E and D, vs A). Image analysis showed a 75% and 95% increase in the apoptotic index by UCNR and BCNR, respectively (bottom, B and D vs A), and treatment with tadalafil reduced these values to a level not significantly different from those in group A. PCNA immunostaining was used to determine corporal cell proliferation, indicating considerably less after BCNR than in group A, which was counteracted by daily tadalafil treatment (Fig. 6 top micrographs, E and D, vs A). UCNR and BCNR led to a 73% and 70% reduction in the replication index compared with group A (middle, B and D, vs A), and tadalafil (E and C) increased those values above those in group A. As a result of these changes, the ratio between the replication and apoptotic indexes was reduced by >80% by both UCNR and BCNR (bottom, B and D vs A), and tadalafil partly counteracted this reduction (C and E).

As we did not use dual immunohistochemistry for ASMA and TUNEL or PCNA, it is not possible to determine whether the changes in cell apoptosis and

*FIG. 6. Effect of long-term treatment with a single daily dose of tadalafil on cell proliferation and turnover in the rat corpora cavernosa. Top panels: adjacent tissue sections to those in the preceding figures were submitted to PCNA immunostaining (×200, bar = 100 μm, the arrows indicate PCNA-positive cells), and the ratio between the proliferation index and the apoptotic index calculated from Fig. 5 was plotted (bottom bar plots). The micrographs depict representative fields and the bar plots show the quantitative image analysis. Groups and P values as in Fig. 1.*

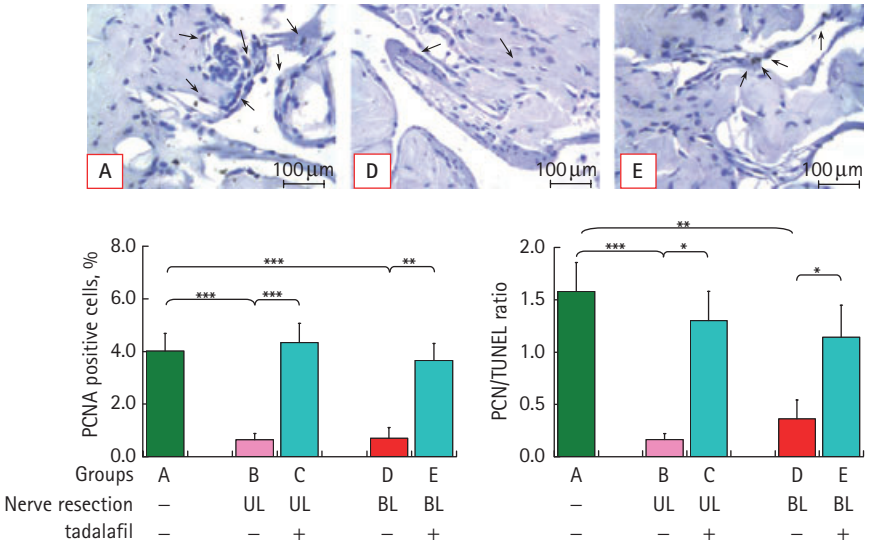
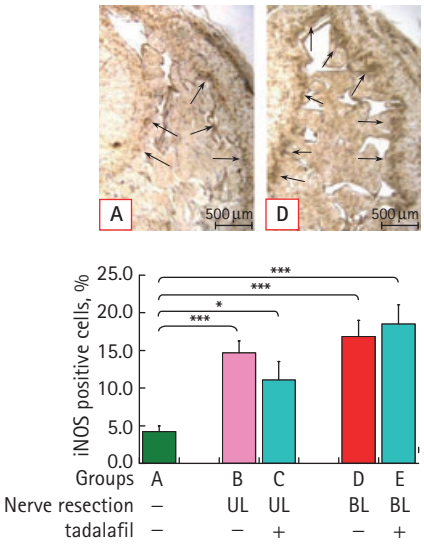



*FIG. 7. Effect of long-term treatment with a single daily dose of tadalafil on iNOS expression in the rat corpora cavernosa. Adjacent tissue sections to those in the preceding figures were immunostained for iNOS. The micrographs show representative fields (×40, bar = 500 μm, the arrows indicate iNOS-positive areas) and the bar plots show the quantitative image analysis. Groups and P values as in Fig. 1.*




proliferation occurred in the SMC, but the location of this staining around the cisternae suggests that this was mainly the case, as SMC are the main cellular component in the corpora cavernosa.

Fibrosis of the corpora cavernosa induced by CNR was accompanied by an increased expression of some fibrotic and antifibrotic factors which are not affected by long-term daily treatment with tadalafil. BCNR led, as expected, to a considerable increase in the expression of one of the main pro-fibrotic factors, TGFβ1, but tadalafil treatment did not reduce this level, as shown in representative views (Fig. 7 top micrographs, D and E vs A). Image analysis indicated that UCNR and BCNR led to a similar increase in TGFβ1 expression, of 75–90%, as in group A (B and D vs A), but tadalafil had little effect on these values (C and E). Similarly, xanthine oxidoreductase, a marker for oxidative stress in corporal tissue, was only moderately but not significantly increased by BCNR, and was not significantly changed by tadalafil, as evaluated by quantitative immunohistochemistry (not shown).

The increase in TGFβ1 by BCNR was paralleled by a very considerable induction of iNOS, a putative antifibrotic factor, and this process was not reduced by tadalafil (Fig. 7 bottom micrographs, D and E vs A). BCNR and UCNR actually increased iNOS by 3–4 times (D and B vs E), but tadalafil had no effect on the expression of iNOS (E and C).

## DISCUSSION

The present results support and extend our previous results in rats subjected to cavernosal nerve damage but treated long-term and continuously with the short-acting PDE5 inhibitors, vardenafil [15] and sildenafil [16], given in the drinking water, showing that long-term treatment with a single daily dose of the long-acting PDE5 inhibitor, tadalafil, induces a similar effect, i.e. the CVOD and underlying histological changes induced by the neuropraxia can be prevented. Our functional results agree with the findings in men after NSRRP treated with long-term daily sildenafil [7] and with the recovery of SM content by a similar treatment [10], and suggest how preventing corporal fibrosis might underlie the recovery of erectile function reported in the human study.

Our results from this rat model suggest that prolonged treatment with single frequent doses of tadalafil might be considered in the clinical setting to preserve the detrimental effects of RP on corporal tissue. Whether tadalafil is given daily, as in the current study, or more sporadically, based on the 3 day-efficacy of tadalafil due to its long half-life [23,24], needs to be determined. The latter might be more practical than for the other PDE5 inhibitors, although sildenafil taken every other night has been shown, as noted, to preserve corporal histology in men after RP [10]. Although the daily doses of vardenafil, sildenafil and tadalafil given in these rat experiments were 2–2.5 times higher (when corrected by surface area) than the usual or accepted daily dosage normally given to men for the on-demand treatment of erectile dysfunction [23,24], no side-effects of the drugs were seen in any of these studies.

The experimental BCNR rat model selected for this and our previous studies using vardenafil and sildenafil represents an extreme condition of nerve injury in which the CNs are resected rather than simply damaged and left *in situ* [12–16]. We chose this method of injury to assure reproducibility among rats, as it is less likely to vary among different laboratories than would any of the other models of crush

injury described previously. In addition, any favourable response to PDE5 inhibitor treatment in the BCNR model suggests that the beneficial effects might even be enhanced, or require lower doses, in a less severe nerve injury that might occur experimentally with the crush models or even in the clinical setting. However, this would also have to be validated to answer at least three questions: (a) is treatment that is initiated after the consequences of the RP-induced neuropraxia on erectile function and corporal smooth muscle become evident, effective in correcting rather than preventing those changes; (b) for how long can treatment be interrupted once normal function is achieved without the risk of a relapse; and (c) are these effects limited to corporal fibrosis, or might the PDE5 inhibitors also facilitate CN regeneration by an alternative mechanism?.

Interestingly, the results obtained previously with vardenafil and sildenafil [15,16], and now tadalafil, given under two different regimens, are qualitatively equivalent in terms of preventing CVOD and the underlying histological alterations. The values of the 'drop rates' determined by cavernosometry in BCNR rats subjected to treatment were identical and in the normal range, as in group A, thus indicating that the CVOD induced by CNR was corrected to the same extent, albeit that vardenafil was slightly less effective in restoring a normal response to intracavernosal papaverine.

The findings were similar for the normalization of the collagen III to I ratio, and for the lack of a significant effect on iNOS expression. However, the single daily dose of tadalafil was more effective than sildenafil in reducing collagen deposition (the effects of vardenafil on collagen deposition were not measured) and stimulating cell proliferation, but was less effective in normalizing ASMA expression or reducing apoptosis. The overall cell turnover measured by the proliferation/apoptosis ratio was protected to the same extent by the three drugs. Finally, tadalafil in this regimen did not reduce the TGFβ1 level, whereas sildenafil did (the assay was not used in the vardenafil study). TGFβ1 does not seem to be in the BCNR/UCNR corpora cavernosa as a critical pro-fibrotic factor, as seen in animal models of diabetes [29,30], or in the tunica albuginea in Peyronie's disease [17,18,20,31,32], or even in the vagina of the diabetic rat [21,33].

As to the mechanism of the effect of long-term PDE5 inhibitors in protecting the corporal cellular/extracellular balance and hence compliance of the corpora, it is most likely due to cGMP stimulating SMC replacement and reducing collagen synthesis via phosphokinase G activation [34–37], rather than acting via iNOS induction. Our previous studies showed that iNOS is used as an endogenous cellular defence to counteract fibrosis, as L-NIL (an inhibitor of iNOS activity) increases CVOD and corporal fibrosis in BCNR [16]. Further proof of this antifibrotic effect of iNOS is seen in the iNOS knockout mouse, where there is an increase in fibrosis in many organs, including the kidney and liver, and even in the aged penis [38–40]. This antifibrotic effect of iNOS would occur by the two processes mentioned for cGMP, either directly through NO release or indirectly through cGMP synthesis, plus the reduction of profibrotic reactive oxygen species in oxidative stress.

Despite the evidence cited above, the relative lack of effect of long-term PDE5 inhibitors on iNOS induction in the corpora of the BCNR rats, as reported here and in a previous study [16], and particularly the previous evidence that L-NIL does not significantly reduce the protective effects of long-term sildenafil on erectile function and the SMC/collagen ratio, make it unlikely that the protective effects of PDE5 inhibitors on the corpora would involve iNOS induction, at least in the BCNR rat model. This would differ from other conditions, like diabetes [29], where oxidative stress in the corpora is more significant than in BCNR. Therefore, in CN damage, the pharmacological increase of cGMP levels by PDE5 inhibitors and the endogenous iNOS induction might act in tandem. However, the latter process does not seem to be essential for maintaining sufficient cGMP to counteract fibrosis in the presence of PDE5 inhibition, as cGMP can be formed from many sources other than via NO from iNOS.

In conclusion, long-term treatment with a single daily dose of tadalafil appears to be as effective as the continuous treatment with vardenafil or sildenafil in preventing CVOD and corporal fibrosis after CN damage, and has more direct clinical relevance. Further studies are needed to decide whether this is related to the long pharmacological half-life of tadalafil, or if the same effects can be achieved with a similar regimen with vardenafil or sildenafil.

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## CONFLICT OF INTEREST

Nestor Gonzalez-Cadavid was the recipient of an investigator initiated grant from Lily ICOS Corp.

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Abbreviations: **ASMA**, α-smooth muscle actin; **(B)(U)CN(R)**, (bilateral) (unilateral) cavernosal nerve (resection); **CVOD**, corporal veno-occlusive dysfunction; **GAPDH**, glyceraldehyde phosphate dehydrogenase; **ICP**, intracavernosal pressure; **(i)NO(S)**, (inducible) nitric oxide (synthase); **PCNA**, proliferating cell nuclear antigen; **PDE5**, phosphodiesterase 5; **SM(C)**, smooth muscle (cells); **TUNEL**, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling; **(NS)(R)RP**, (nerve-sparing) (retropubic) radical prostatectomy.

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REVIEW

# Rationale for phosphodiesterase 5 inhibitor use post-radical prostatectomy: experimental and clinical review

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**Erectile dysfunction (ED) is a common complication after radical prostatectomy and results from trauma sustained by the cavernosal nerves. This is a major concern for patients and often affects treatment decisions. The likely mechanism for post-prostatectomy ED is through corporal veno-occlusive dysfunction. There is an increasing amount of evidence to suggest that phosphodiesterase 5 inhibitors (PDE5 inhibitors), when given on a continuous long-term basis, can help to prevent and reverse ED after surgery. In this review article we will examine the pathophysiology of post-prostatectomy ED and discuss the experimental and available clinical evidence for administering PDE5 inhibitors after prostatectomy.**
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**Keywords:** prostatectomy; erectile dysfunction; phosphodiesterase 5 inhibitors

## Erectile dysfunction is common following radical prostatectomy

Recent data suggest that approximately 161 000 men per year undergo a radical prostatectomy in the United States.[1] It is thought that many more men who are diagnosed with early-stage prostate cancer would accept this surgical form of treatment for their newly diagnosed disease if it were not for the possibility of the development of erectile dysfunction (ED). This common complication after radical prostatectomy is largely due to trauma sustained by the cavernosal nerves and is still widely encountered even after the most recent advances in surgical technique to spare the nerves.[2–5] Of men that are potent before surgery, only about 40–74% of men regain sexual function.[4–7] In fact, 41.9% of men reported that their sexual performance was a moderate to large problem after surgery[7] and patients appear to value sexual function so highly that they are often willing to choose therapy that offers better potency with lower life expectancy than options that offer longer life expectancy and lower potency rates.[8]

Radical prostatectomy seems to disrupt and/or damage the neurovascular mechanisms responsible for eliciting an erection thereby resulting in either temporary or permanent ED postoperatively. In its mildest form, apparent in a successful bilateral nerve sparing prostatectomy, the trauma resulting from surgical manipulation of the neurovascular bundles may result in a neuropraxia leading to temporary ED. The most severe form of ED results from a non-nerve sparing prostatectomy where both nerves are transected, either volitionally or unrecognized at the time of surgery, and this leads to the complete loss of neuroregulatory functions in the corpora cavernosa.

## CVOD is the most common form of ED following radical prostatectomy

Injury to the cavernosal nerves results in the atrophy and degradation of the underlying cavernosal smooth muscle, which, besides resulting in ED, may also lead to a decrease in penile weight.[9] Histologically, such a neuropraxia/neurotomy leads to apoptosis of the cavernosal smooth muscle and an excessive deposition of collagen within the cavernosa, which clinically results in corporal

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veno-occlusive dysfunction (CVOD). CVOD or venous leakage occurs because of the inability of the cavernosal smooth muscle cell mass to adequately compress the subtunical veins and prevent leakage of blood out of the cavernosa during tumescence. With CVOD, the patient complains of the inability to obtain and maintain an erection sufficient for completion of the sexual act and CVOD has been recognized as the major cause of ED subsequent to radical prostatectomy.[10,11]

Therefore, the poor response of many patients to the oral phosphodiesterase 5 inhibitors (PDE5) inhibitors given on demand post-radical prostatectomy could be due either to the neural injury which prevents the normal release of nitric oxide from the cavernosal nerve endings (a necessary requirement for the synthesis of the second messenger cyclic guanosine monophosphate (cGMP) within the cavernosa) or the subsequent loss of some of the corporal smooth muscle mass as elucidated above due to the neural injury or a combination of both conditions. In addition, the failure of vasoactive drugs injected intracorporeally into the penis to induce an erection in post-prostatectomy patients suggests that the corporal smooth muscle mass has most likely been impacted by the surgery in these patients. While arterial insufficiency post-prostatectomy due to intraoperative damage to the arteries supplying blood to the cavernosa is another means by which some of these men may experience ED,[12] it is CVOD that is the predominant cause of ED post-prostatectomy.

Although the hypoxia theory has been promulgated as the reason why there is a loss of corporal smooth muscle and an increase in collagen following this neural injury post-prostatectomy, the scientific evidence to show that intracellular hypoxia occurs during detumescence is very weak at best. This hypoxia theory states that low-oxygen tension occurs within the cavernosal tissue when there is detumescence and this leads to the induction of elevated levels of the profibrotic cytokine, transforming growth factor-$\beta$1 (TGF$\beta$1), within the cavernosa.[13,14] However, to date, the only p02 that has been measured within the penis in such a setting is the sinusoidal p02 and there is no scientific evidence that the smooth muscle and other components of the cavernosal tissue obtain $O_2$ from the sinusoids rather than their own capillaries.

## Antifibrotic role of NO following cavernosal nerve resection

The one theory that seems to be the most plausible in explaining why the corporal smooth muscle deteriorates in tandem with an increase in collagen content following radical prostatectomy is that it is the neural injury itself that induces proapoptotic (loss of smooth muscle) and profibrotic (increase in collagen) factors within the cavernosa. It is possible that the ablation by neuropraxia of certain key growth factors produced by the cavernosal nerves may be responsible for eliciting the smooth muscle fibrosis and atrophy observed in corporal tissue. However, the production of cytokines and noxious agents by the damaged nerve axons may also be the causal factor of the increased early smooth muscle apoptosis,[15,16] which in turn may trigger collagen deposition to replace the lost cells. Similarly to the situation with skeletal muscle atrophy after denervation,[17,18] the molecular and cellular etiology of the tissue atrophy subsequent to cavernosal nerve damage remains to be elucidated.

In an attempt to counteract this proapoptotic and profibrotic cascade induced by such a neural injury, the cavernosal tissue itself then initiates an antiapoptotic and an antifibrotic defense mechanism via the formation of nitric oxide and cGMP within the smooth muscle itself (Figure 1).[19] The key to erectile function post-radical prostatectomy in patients who are potent before the prostatectomy is maintenance of the integrity of the corporal histology (that is, prevention of both fibrosis and apoptosis of the smooth muscle). Therefore, in the post-prostatectomy patient, tumescence should be attainable au natural if both the nerves and blood vessels are not injured during the surgery. However, even if the nerves are injured but the histology of the cavernosal tissue can be preserved, as long as the arterial inflow is not impeded then tumescence in these patients may be achieved albeit with the use of intracorporeal injections and possibly with intraurethral applications of vasoactive substances.

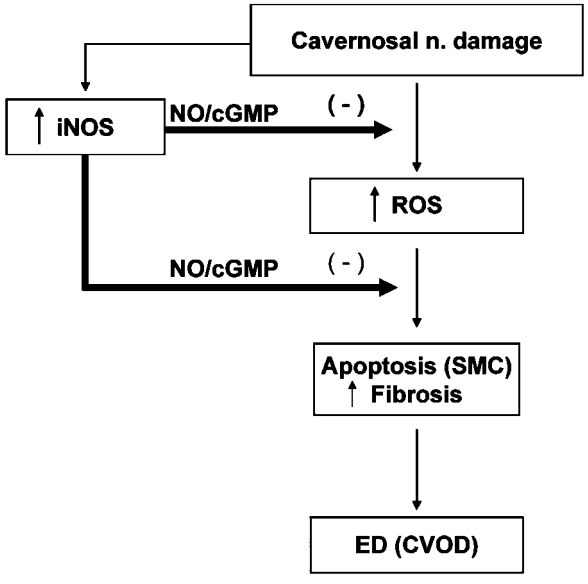


**Figure 1** Effect of nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) from inducible isoform of nitric oxide synthase (iNOS) in combating reactive oxygen species (ROS), fibrosis and apoptosis. (–), inhibitory effect.

Nitric oxide, as the case of its subsequent downstream second messenger cGMP that also acts as an antifibrotic agent in the setting of cavernosal nerve injury, does not emanate from the nitrergic nerve endings of the cavernosal nerve but is induced by the smooth muscle itself.[18] The nitric oxide from the nerve endings of the cavernosal nerve is produced by the neuronal isoform of the nitric oxide synthase (nNOS) enzyme[20] whereas the nitric oxide that emanates from the cavernosal smooth muscle cells once the neural injury occurs is derived, at least in part, from the induction of the inducible isoform of nitric oxide synthase (iNOS).[15,16] There is a marked distinction between these two isoforms. While nitric oxide in the corpora during sexual stimulation is believed to be produced immediately upon sexual stimulation, albeit in small amounts, primarily by the activation of nNOS, the production of nitric oxide from iNOS in the corpora is very different from that of nNOS in that it is unrelated to sexual stimulation and occurs by transcriptional induction that results in the production of sustained amounts of nitric oxide, although somewhat delayed in its onset.

The evidence supporting the view that iNOS undergoes spontaneous induction in the corpora cavernosa in certain conditions such as aging, diabetes and specifically cavernosal nerve damage by protecting the histological and functional integrity of the corpora through combating fibrosis, stems from four main sources of experimental data. First, the fact that general inhibition of the activity of all NOS isoforms, and hence nitric oxide production, by prolonged sustained administration of N(G)-nitro-L-arginine methyl ester to rats, leads to considerable fibrotic degeneration in organs such as the heart, liver and kidney, independent of hemodynamic factors that may contribute to this process.[21–25] Second, specific genetic blockade of iNOS in the iNOS knockout mice leads to exacerbation of experimental fibrosis of the kidney and liver,[26,27] and the chronic inhibition of iNOS activity in rats by N6-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL) intensifies aging-related fibrosis of the arterial wall and of the experimentally induced Peyronie's disease-like fibrotic plaque in the penis.[28,29] Third, administration of iNOS cDNA in the latter model reduces the fibrotic plaque.[30] Finally, specifically in cavernosal nerve damage, a similar treatment with L-NIL exacerbates CVOD and the underlying fibrosis of the corpora.[16]

It is assumed that prolonged endogenous induction of iNOS to moderate levels produces sufficient nitric oxide as to reduce collagen synthesis, quench reactive oxygen species (ROS), inhibit TGF$\beta$1 expression and myofibroblast differentiation, and activate metalloproteinases that break down collagen.[17] If nitric oxide reaches excessive levels it may turn to be deleterious by causing cell death and oxidative stress, and this will depend on the tissue environment.

## PDE5 inhibitors protect the integrity of the corporal smooth muscle following cavernosal nerve resection: experimental evidence

There is quite an amount of emerging scientific evidence to suggest that prolonged elevated levels of nitric oxide and cGMP can have an antifibrotic effect on a variety of tissues including tunica albuginea and corporal tissue (Figure 2).[19,20,31] Since PDE5 inhibitors work by inhibiting the enzyme that degrades cGMP, and since cGMP via activation of PKG inhibits collagen synthesis, this may be the preferential route of antifibrotic action when cGMP levels are maintained high for sustained periods. Although cGMP seems to stabilize iNOS mRNA or activate its transcription,[32] and thus may upregulate nitric oxide production, recent evidence suggest that this is not the case for the long-term effects of tadalafil on corporal fibrosis after cavernosal nerve damage in the rat.[16] Although there have been reports in the literature regarding the clinical effects of administration of these PDE5 inhibitors post-prostatectomy,[33–36] the rationale behind the use of these drugs on a prolonged and continuous basis in the post-prostatectomy patient has never been fully and scientifically delineated. The recent publication by Ferrini *et al.*[15] examined the effects of the administration of the PDE5 inhibitor, vardenafil, and demonstrated that the prolonged and continuous administration of the compound was effective in preventing both the fibrosis and loss of smooth muscle seen following bilateral cavernosal nerve resection (Figure 3). Compared with the sham group, the bilateral cavernosal nerve resection rats demonstrated a threefold increase in intracorporeal apoptosis, a 60% reduction in the smooth muscle to collagen ratio, a twofold increase in iNOS expression and development of CVOD (Table 1). When vardenafil was given daily for 45 days to the animals that underwent bilateral cavernosal nerve resection, CVOD did not develop and the abnormal corporal smooth muscle to collagen ratio seen in the bilateral cavernosal nerve-resected group was normalized. Similar results have been reported in the both the unilateral and bilateral nerve resection

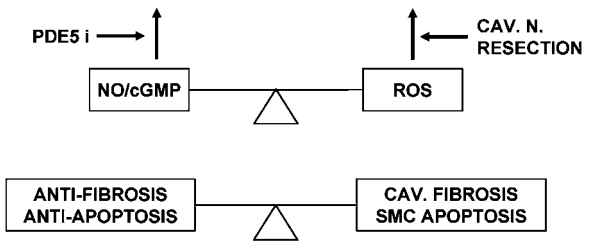


**Figure 2** Relationship between reactive oxygen species (ROS) and nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) following cavernosal nerve resection.

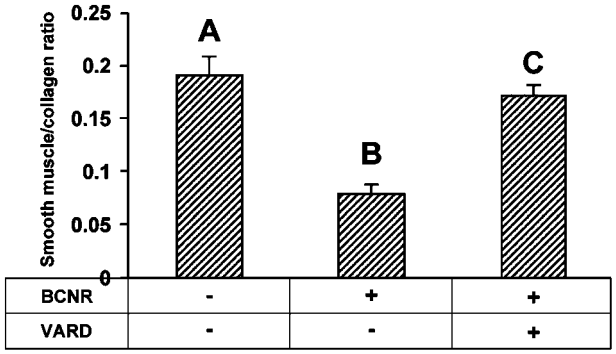


**Figure 3** Effect of bilateral cavernosal nerve resection (BCNR) and vardenafil treatment on the smooth muscle/collagen ratio in the rat corpora cavernosa. Bilateral cavernosal nerve resection leads to a decrease in the smooth muscle/collagen ratio, which leads to the development of CVOD. The ratio becomes similar to the control group after treatment with vardenafil. Values determined by quantitative image analysis. BCNR (**b**) vs sham (**a**) or BCNR + V (**c**): $P<0.001$; sham (**a**) vs BCNR + V (**c**): not significant. Ferrini *et al*.[15] with permission.

**Table 1** Dynamic infusion cavernosometry

| | *ICP after papaverine (mm Hg + s.e.m.)* | *Drop rate (mm Hg/min)* |
|---|---|---|
| Control ($n=6$) | 62 ± 8 | 18 ± 2 |
| Bilateral nerve resection ($n=7$) | 64 ± 6 | 42 ± 5** |
| Bilateral nerve resection + vardenafil ($n=11$) | 61 ± 2 | 17 ± 2* |

Abbreviation: ICP, intracavernosal pressure.
Dynamic infusion cavernosometrics in the rat after bilateral cavernosal nerve resection with and without continuous daily vardenafil for 45 days.
Drop rate was measured as the decrease in intracavernosal pressure in 1 min after cessation of saline infusion into the penis. This implies that the bilateral nerve-resected group has a large drop rate suggestive of a venous leak.
When treated with vardenafil, the drop rate is similar to the control group, thus suggesting that the CVOD has been normalized.
*Denotes $P<0.05$ compared to BCNR and ** compared to sham operated. Ferrini *et al*.[15] with permission.

models using continuous long-term administration of sildenafil.[16]

A parallel study by Vignozzi *et al*.[37] also found that chronic tadalafil administration (120 days) to rats similarly reversed the decline in the cavernosal smooth muscle to collagen ratio that occurred after a bilateral cavernous neurotomy. Although Vignozzi *et al*. hypothesized that the effect of the tadalafil may have been due to the reversal of hypoxia induced by the neurotomy, these studies taken together suggest that PDE5 inhibitors can potentially protect and preserve the integrity of the corpora after cavernosal nerve damage when given on a prolonged and continuous basis.

## PDE5 inhibitors protect the integrity of the corporal smooth muscle following cavernosal nerve resection: clinical evidence

The only clinical trial to suggest vaguely that chronic PDE5 inhibition post-prostatectomy may preserve the integrity of the corpora emanates from Schwartz *et al*.[35] who performed post-prostatectomy biopsies on men on chronic sildenafil treatment and found that the corporal smooth muscle to collagen ratio was maintained in those patients treated with chronic sildenafil while those who did not take sildenafil showed loss of smooth muscle content with a concomitant increase in collagenization of the corpora.

Clinical trials have also examined whether the routine use of PDE5 inhibitors on an on-demand basis to induce an erection post-prostatectomy, as opposed to its chronic use on a daily basis, may be beneficial long term in treating or correcting the ED that occurs after radical prostatectomy. Indeed, there are a number of such on-demand treatment studies using each one of the PDE5 inhibitors, vardenafil,[33] sildenafil[34] and tadalafil,[36] and each one touting the efficacy of their compound in improving post-prostatectomy potency rates. Although the theory promulgated by these latter nonrandomized, non-controlled studies to explain the efficacy of the PDE5 inhibitors is via cavernosal oxygenation, as enumerated earlier in this review there is as yet no direct scientific evidence to support that these compounds improve tissue oxygenation within the corporal tissue itself.

What is apparent from the emerging clinical and experimental data on the use of PDE5 inhibitors post-prostatectomy is that these drugs appear to play some role in preserving the integrity of the corporal tissue following cavernosal nerve damage (Figure 3). The importance of this observation is that regardless of whether the neural injury to the penis following surgery is permanent or not, preservation of the normal histology of the preoperative cavernosa will allow the corporal tissue to respond normally to the administration of locally administered proerectogenic agents even if the tissue should fail to respond normally to the on-demand agents. Emerging studies focusing on the molecular mechanisms of apoptosis and fibrosis are beginning to shed some light as to why the chronic and prolonged use of PDE5 inhibitors is proving to be beneficial. Obviously, further animal and randomized clinical studies are needed to confirm these exciting preliminary observations. A paradigm involving the discontinuation of the PDE5 inhibitor administration after the long-term post-prostatectomy treatment would allow one to verify whether indeed the improved erectile response is maintained in the total absence of the drug. The latter would suggest that the beneficial effects of this regimen with PDE5

inhibitors on the underlying corporal histology seen in the rat model would also occur in men, and support the role of smooth muscle fibrosis in the etiology of CVOD.

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32 Bojunga J, Dresar-Mayert B, Usadel KH, Kusterer K, Zeuzem S. Antioxidative treatment reverses imbalances of nitric oxide synthase isoform expression and attenuates tissue-cGMP activation in diabetic rats. *Biochem Biophys Res Commun* 2004; **316**: 771–780.
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34 Lowentritt BH, Scardino PT, Miles BJ, Orejuela FJ, Schatte EC, Slawin KM *et al.* Sildenafil citrate after radical retropubic prostatectomy. *J Urol* 1999; **162**: 1614–1617.
35 Schwartz EJ, Wong P, Graydon RJ. Sildenafil preserves intracorporeal smooth muscle after radical retropubic prostatectomy. *J Urol* 2004; **171**: 771–774.
36 Montorsi F, Nathan HP, McCullough A, Brock GB, Broderick G, Ahuja S *et al.* Tadalafil in the treatment of erectile dysfunction following bilateral nerve sparing radical retropubic prostatectomy: a randomized double-blind, placebo controlled trial. *J Urol* 2004; **172**: 1036–1041.
37 Vignozzi L, Filippi S, Morelli A, Ambrosini S, Luconi M, Vannelli GB *et al.* Effect of chronic tadalafil administration on penile hypoxia induced by cavernous neurotomy in the rat. *J Sex Med* 2006; **3**: 419–431.

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

| NAME GONZALEZ-CADAVID, NESTOR F | POSITION TITLE Professor |
|---|---|
| eRA COMMONS USER NAME (credential, e.g., agency login) NESTORGON | |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| University of Buenos Aires, Argentina | M.Sc. | 1961 | Biochemistry |
| University of Buenos Aires, Argentina | Ph.D. | 1964 | Biochemistry |
| University of London, England | Ph.D. | 1967 | Biochemistry |

### A. Positions and Honors.

1961 Gold medal to the best MSc graduate, University of Buenos Aires
1961-62 Fellowship, National Council of Scientific Research (Argentina)
1964 Gold Medal to the best doctorate dissertation, University of Buenos Aires
1964-66 Fellowship, Natl Council Sci Res (Argentina), work at the Courtauld Inst Biochemistry, London Univ.
1967 Fellowship, WelcomeTrust (England), ibid
1968-71 Assoc. Professor, Dept. Biochemistry., Sch. of Science, Central University, Caracas, Venezuela
1971-92 Full Professor, Dept Cell Biology, School of Science, Central University, Caracas, Venezuela
1978-79 Gosney Visit. Assoc. in Biology, California Institute of Technology, Biology Division, Pasadena, CA
1980 Senior Fellowship, Guggenheim Foundation, Cal. Inst. of Technology, Biology Div., Pasadena, CA
1982 Visiting Professor, University of Buenos Aires, School of Biochemistry, Buenos Aires, Argentina
1984 Fellowship, Internatl Union Against Cancer, City of Hope Med Center, Div Biology, Duarte (CA).
1987-88 Visiting Professor, UCLA School of Medicine, Div of Hematology/Oncology, Los Angeles, CA
1987 E. Roosevelt fellowship, Internatl Union Against Cancer, UCLA Med School, Dept Medicine, Los Angeles (CA); Senior Fellowship, United Nations Univ. ibid
1990-92 National Research Service Award (Senior Fellowship), Popul. Res. Center, Harbor/UCLA Med. Ctr.
1990-96 Adj. Associate Professor, Dept of Surgery/Urology, UCLA School of Medicine, Director Urology Research Laboratory, Harbor-UCLA REI
1996-on Adjunct Professor, Department of Urology, UCLA School of Medicine, and Director, as above
1997-on Professor, Dept of Internal Medicine/Endocrinology, Charles R. Drew University.
2001-on Director, RCMI Molecular Medicine Core, Charles R. Drew University

### B. Professional membership

1965-75 Bioch Soc (England); 1987-90 Tissue Cult Assoc (USA); 1989 Amer Assoc for Cancer Res.; 1992-Am Soc Andrology (USA): 1998-Endocrinol Society (USA); 2000 Am Urological Assoc (USA); 2000 Soc Study Impotence; 2000 Soc Study Reproduction; 2004 Sexual Medicine Soc of N Am

### C. Selected peer-reviewed publications from 2003-2007 (from a list of 154 on CV)

Ferrini MG, Magee TR, Vernet D, Rajfer J, **Gonzalez-Cadavid NF** (2003) Penile neuronal nitric oxide synthase (PnNOS) and its regulatory proteins are present in hypothalamic and spinal cord regions involved in the control of penile erection. J Compar Neurol 458:46-61

Magee T, Zeller CB, Ferrini M, Davila H, Vernet D, Burnett AL, Rajfer J, **González-Cadavid NF** (2003) A protein inhibitor of NOS (PIN) is expressed in the rat and mouse penile nerves and co-localizes with penile neuronal NOS (PnNOS) Biol Reprod 68:478-488.

Berman JR, Almeida FG, Jolin J, Raz S, Chaudhuri G, **Gonzalez-Cadavid NF** (2003) Androgen receptors, aromatase, and 5--reductase in human vagina: correlation with menopausal status. Fertil Steril, 79:925-931.

Davila H, Ferrini M, Rajfer J, **Gonzalez-Cadavid NF** (2003) Fibrin induction of a Peyronie's-like plaque in the rat penile tunica albuginea. A new model for Peyronie's disease. Br J Urol, 91:830-838.
Reisz-Porszasz S, Bhasin S, Artaza JN, Shen R, Sinha-Hikim I, Houge A, **Gonzalez-Cadavid NF** (2003) Reduction of skeletal muscle mass in a transgenic mouse that hyperexpresses myostatin in the muscle. Am J Physiol, Am J Physiol, 2003 Oct;285(4):E876-888.
Ma K, Mallidis C, Bhasin S, Mahabadi V, Artaza J, **Gonzalez-Cadavid N**, Arias J, Salehian B (2003) Glucocorticoid-Induced Skeletal Muscle Atrophy is Associated with Upregulation of Myostatin Gene Expression. Am J Physiol, 285:E363-371.
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Singh R, Artaza JN, Taylor WE, (**Gonzalez-Cadavid NF***, Bhasin S*; *: equal contributors) (2003) Androgens stimulate myogenenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent stem cells through an androgen receptor-mediated pathway. Endocrinology, 144:5081-508
Liu W, Thomas SG, Asa SL, **Gonzalez-Cadavid NF**, Bhasin S, Ezzat S (2003) Myostatin is a Skeletal Muscle Target of Growth Hormone Anabolic Action. J Clin Endocrin Metabol, 88:5490-5496
Bhasin S, Taylor W, Singh R, Artaza J, Sinha-hickim I, Jasuja R, Choi H, **Gonzalez-Cadavid NF** (2003) The mechanisms of androgen effects on body composition: mesenchymal pluripotent cell as the target of androgen action. J Gerontol, 58:M1103-10.
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Davila HH, Magee TR, Rajfer J, **Gonzalez-Cadavid NF** (2004) Gene therapy with the inducible nitric oxide synthase (iNOS) cDNA regresses the fibrotic plaque in an animal model of Peyronie's disease**.** Biol Reprod, 71:1568-1577
Davila HH, Rajfer J, **Gonzalez-Cadavid NF** (2004) Corporal veno-occlusive dysfunction in the aging rat. evaluation by cavernosometry and cavernosography. Urology, 64:1261-1266.
**Gonzalez-Cadavid NF**, Rajfer J (2004) Molecular pathophysiology and gene therapy of aging-related erectile dysfunction. Exptl Gerontol, 39:1705-1712
Sinha-Hikim I, Taylor WE, **Gonzalez-Cadavid NF**, Zheng W, Bhasin S (2004) Androgen receptor in human skeletal muscle and cultured muscle satellite cells: Up-regulation by androgen treatment. J Clin Endocr Metabol 89:5245-5255.
Davila H, Magee TR, Rajfer J, **Gonzalez-Cadavid NF** (2005). Peyronie's disease is associated with an increase of plasminogen activator inhibitor-1 (PAI-1) at the RNA and protein levels. Urology. 65:645-648
Hikim AP, Vera Y, Vernet D, Castanares M, Diaz-Romero M, Ferrini M, Swerdloff RS, **Gonzalez-Cadavid NF**, Wang C (2005) Involvement of nitric oxide-mediated intrinsic pathway signaling in age-related increase in germ cell apoptosis in male brown-norway rats. J Gerontol A Biol Sci Med Sci 60:702-708.
Artaza JN, Bhasin S, Magee TR, Reisz-Porszasz S, Shen R, Groome NP, Fareez MM, **Gonzalez-Cadavid NF** (2005) Myostatin inhibits myogenesis and promotes adipogenesis in C3H 10T(1/2) mesenchymal multipotent cells. Endocrinology 146:3547-3557.
Vernet D, Qian A, Nolazco G, Cantini L, Magee TR, Ferrini MG, Rajfer J, **Gonzalez-Cadavid NF** (2005) Evidence that osteogenic progenitor cells in the human tunica albuginea may originate from stem cells. Implications for Peyronie's disease. Biol Reprod, 73:1199-1210.
Jasuja R, Ramaraj P, Mac RP, Singh AB, Storer TW, Artaza J, Miller A, Singh R, Taylor WE, Lee ML, Davidson T, Sinha-Hikim I, **Gonzalez-Cadavid N**, Bhasin S (2005) Delta-4-androstene-3,17-dione binds androgen receptor, promotes myogenesis in vitro, and increases serum testosterone levels, fat-free mass, and muscle strength in hypogonadal men. J Clin Endocrinol Metab. 90:855-863.
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Kovanecz I, Ferrini MG, Vernet D, Nolazco G, Rajfer J, **Gonzalez-Cadavid NF** (2006). Pioglitazone prevents corporal veno-occlusive dysfunction (CVOD) in a rat model of type 2 diabetes melittus. BJU Int, 98:116-124
Ferrini MG, Davila H, Kovanecz I, Sanchez S, **Gonzalez-Cadavid NF*** Rajfer J* (*: equal contributors) (2006**)** Long-term continuous treatment with vardenafil prevents fibrosis and preserves smooth muscle content in the rat corpora cavernosa after bilateral cavernosal nerve transection. Urology, 2006 Aug;68(2):429-35.
Ferrini MG, Kovanecz I, Sanchez S, Vernet D, Davila HH, Rajfer J, **Gonzalez-Cadavid NF** (2007) Long-term continuous treatment with sildenafil ameliorates aging-related erectile dysfunction and the underlying corporal fibrosis. Biol Reprod, 76:915-923
Artaza JN, Reisz-Porszasz S, Dow JS, Kloner RA, Tsao J, Bhasin S, **Gonzalez-Cadavid NF** (2007) Alterations in myostatin expression are associated with changes in cardiac left ventricular mass but not ejection fraction in the mouse. J Endocrinol, 194(1):63-76.
**Gonzalez-Cadavid NF,** Rajfer J (2007) Experimental models for the study of the cellular and molecular pathophysiology of Peyronie's disease. In: Current Clinical Urology: Peyronie's Disease, A Guide to Clinical Management, ed by L.A. Levine, Humana Press, Totowa, NJ, p 19-39
Magee TR, Kovanecz I, Davila HH, Ferrini MG, Cantini L, Vernet D, Zuniga FI, Rajfer J, **Gonzalez-Cadavid NF** (2007) Antisense and short hairpin RNA (shRNA) constructs targeting PIN (protein inhibitor of NOS) ameliorate aging-related erectile dysfunction in the rat. J Sex Medic, 4(3):633-43.
Kovanecz I, Ferrini MG, Davila HH, Rajfer J, **Gonzalez-Cadavid NF** (2007) Pioglitazone ameliorates penile corpora veno-occlusive dysfunction (CVOD) in the aged rat. BJU Int. 2007 Oct; 100(4):867-74.
Kovanecz I, Rambhatla A, Ferrini MG, Vernet D, Sanchez S, Rajfer J, **Gonzalez-Cadavid NF** (2007) Long term sildenafil treatment ameliorates corporal veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats. Int J Impot Res, 2007, 100(4):867-74.
Kovanecz I, Rambhatla A, Ferrini MG, Vernet D, Sanchez S, Rajfer J, **Gonzalez-Cadavid** NF (2008) Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction (CVOD) that occurs following cavernosal nerve resection in the rat. BJU Int 101(2):203-10.
Rambhatla A, Kovanecz I, Ferrini M, **Gonzalez-Cadavid NF**, Rajfer J (2008) Rationale for phosphodiesterase 5 inhibitor use post-radical prostatectomy: experimental and clinical review. Int J Impot Res. 20(1):30-4.
Nolazco G, Kovanecz I, Vernet D, Ferrini M, Gelfand B, Tsao J, Magee T, Rajfer J, **Gonzalez-Cadavid NF** (2008) Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. BJU Int, in press
Artaza JN, Singh R, Ferrini MG, Braga M, Tsao J, **Gonzalez-Cadavid NF** (2008) Myostatin promotes a fibrotic phenotypic switch in multipotent C3h 10T1/2 cells without affecting their differentiation into myofibroblasts. J Endocrinol 196:235-49
Cantini LP, Ferrini MG, Vernet D, Magee TR, Quian A, Gelfand RA, Rajfer J, **Gonzalez-Cadavid NF** (2008) Pro-fibrotic role of myostatin in Peyronie's disease. J Sex Med, in press

## D. Ongoing Research Support.

1. G12RR030262 (PI: Kelly, Baker; NGC: Core Director) 10/01/00-09/30/05 Resubmitted for 04/01/08-03/31/13
NIH: RCMI Infrastructure Development Grant Drew bridge funding 10/01/05-ongoing
DNA Repository and Molecular Medicine Core. Renewal as "Molecular Medicine and Stem Cells Core".
This is an institutional support that will cease once the RCMI grant is funded

2. RO1 DK53069-07 (PI: Gonzalez-Cadavid), 05/01/03-04/30/08 NIH/NIDDK

Erectile Dysfunction and Nitric Oxide Synthase in Aging
The goal is to apply novel procedures of gene and stem cell therapy for the treatment of aging-related erectile dysfunction, based on the modulation of the nitric oxide/cGMP pathway in the corpora cavernosa in a rat model of reproductive aging, and whether this restores nitrergic neurotransmission and/or corporal smooth muscle

3. 11881-01R (PI: Gonzalez-Cadavid) 08/01/05-07/31/08 American Diabetes Association
Erectile dysfunction and vascular fibrosis in diabetes
The goal is to study whether the pharmacological modulation of the nitric oxide/cGMP pathway in the corpora cavernosa and the arterial wall prevents fibrosis and loss of compliance in an animal model of type 2 diabetes

4. 512190-01 (PI: Gonzalez-Cadavid) 04/01/07-03/31/08 LABioMed Stem cell seed grant
Characterization of stem cells from human skeletal muscle for the therapy of congestive heart failure
The goal is to determine whether skeletal myoblast preparations from patients with congestive heart failure in an ongoing clinical trial contain stem cells and whether they can undergo cardiomyocyte differentiation

5. PR064756 (PI: Gonzalez-Cadavid) 03/01/07-02/28/10 Department of Defense
Pharmacological prevention and reversion of erectile dysfunction after radical prostatectomy, by modulation of nitric oxide/cGMP pathways
The goal is to determine whether long-term treatment with PDE5 inhibitors and nitric oxide donors can prevent corporal veno-occlusive dysfunction in a rat model of erectile dysfunction after radical prostatectomy, and whether this is due to an improvement in the underlying penile corporal fibrosis and loss of smooth muscle

6. PC061300 (PI: Gonzalez-Cadavid) 03/31/07-02/28/11 Department of Defense
Modulation of stem cell differentiation and myostatin as an approach to counteract fibrosis in dystrophic muscle regeneration after injury.
The goal is to determine whether skeletal muscle derived stem cells (MDSC) can ameliorate skeletal muscle atrophy and fibrosis in a mouse model of Duchenne's muscular dystrophy, and this is stimulated by ex vivo gene transfer of myostatin shRNA to stem cells, and/or treatment with agents that inhibit myostatin activity

7. (Gonzalez-Cadavid) 04/01/07- 03/31/08 Takeda North America, Inc
Antifibrotic and Renoprotective Effects of Pioglitazone on Type 2 Diabetes Related Tubulointerstitial Fibrosis
The goal is to determine whether long-term treatment with pioglitazone reduces vascular and renal fibrosis in an animal model of type 2 diabetes

8. Pilot grant (Bathia/Ho/Gonzalez-Cadavid) 04/01/07-03/31/08 Harbor/UCLA Division of Urodynamics
Reversion of levator ani atrophy by muscle derived stem cells in a rat model of stress urinary incontinence
The goal is to determine whether MDSC can replace defective myofibers, and reduce fibrotic degeneration, in the levator ani of a rat model for female stress urinary incontinence (SUI), and correct SUI

9. NIH R21DK070003-01A1 (Gonzalez-Cadavid) 09/01/07-08/31/09 NIH NIDDK
Cell-selective expression of fibrotic gene pathways
The goal is to compare the patterns of gene expression related to fibrotic phenotypes in smooth muscle and fibroblasts in the corpora cavernosa in rat models of reproductive aging and Peyronie's disease, and the relationship between stem cells, smooth muscle cells, and fibroblasts, in myofibroblast generation in fibrosis.

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

| NAME | POSITION TITLE |
|---|---|
| KOVANECZ, ISTVAN | Assistant Professor (appointment in progress) |
| eRA COMMONS USER NAME (credential, e.g., agency login)<br>IKOVANECZ0308 | |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| University of Szeged (former Jozsef A. University of Art and Sciences), Szeged, Hungary | M.Sc. | 1985 | Biochemistry |
| Budapest University of Technology, Institute of Continuing Engineering Education, Budapest, Hungary | CNRT | 1987 | Nuclear technology |
| University of Szeged (former Jozsef A. University of Art and Sciences), Szeged, Hungary | Ph.D | 1994 | Comparative Physiology |
| Biological Research Center of The Hungarian Academy of Sciences, Szeged, Hungary | | 1999-2000 | Genomics, IT, Bioinformatics |

### A. Positions and Honors

1985-1987 Research Fellow, Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary
1987-1991 Research Scientist, Blood Transfusion Center, Szent-Gyorgyi Albert Medical University, Szeged, Hungary
1991-1992 Volunteer Researcher, Department of Neurology, Mount Sinai Medical Center, CUNY, New York, NY, USA
1993-1999 Senior Research Scientist, Head of the Vivarium, Department of Pharmacology and Pharmacotherapy, Szent-Gyorgyi Albert Medical University, Szeged, Hungary
1999-2001 Biologist Chief Counselor, Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary
2000-2001 Member of the Computer Software Council of the Hungarian Academy of Sciences
2004–on Research Associate, Urology Research Laboratory, Department of Surgery, Los Angeles Biomedical Research Institute at Harbor-UCLA, Torrance, CA, USA
2008-on Assistant Professor (in progress), Department of Urology, UCLA David Geffen School of Medicine, Los Angeles, CA, USA

### B. Professional membership

1982–96 John von Neumann Society of Computer Sciences
1993–01 Hungarian Genetical Society
1994–99 Hungarian Society of Cardiology
1994–99 Hungarian Society for Experimental and Clinical Pharmacology
1994–99 International Society for Heart Research (European Section)
2000–01 Computer Software Council of the Hungarian National Academy of Sciences
2006–on National Geographic Society
2007–on Sexual Medicine Society of North America
2008–on American Urological Association

### C. Selected publications

**Original research and theoretical treatises**


1. Bodis-Wollner I, Antal A, **Kovanecz I**. Low-dose scopolamine and acetyl-levo-carnitine dissociate primary from cognitive visual processing in the trained monkey. *Invest Ophth Vis Sci* 1993; 34(4): 1174.
2. Antal A., **Kovanecz I,** Bodis-Wollner I. Visual discrimination and P300 are affected paralell by cholinergic agents in the behaving monkey. *Physiol Behav.* 1994; 56(1) 161-66.
3. Tagliati M., Bodis-Wollner I., **Kovanecz I,** Stanzione P. Spatial frequency tuning in the monkey retina depends on D2 receptor-linked action of dopamine. *Vision Research* 1994; 34(16):22051-57.
4. **Kovanecz I**, Csajbok E., Petri I.B. In vitro steroid sensitivity in chronic uremic and kidney transplant patients: HLA - associated susceptibility to steroid treatment. *Nephrology Dialysis Transplantation* 1994; 9(10): 1474-76.
5. **Kovanecz I**, Petri I.B., Kaiser G. HLA associated lymphocyte panel reactive (cytotoxic) antibody production in dialyzed chronic uremic patients. *Acta Microbiologica Hungarica* 1995; 42(1): 81-84.
6. **Kovanecz I,** Papp JG., Szekeres L. Increased cardiac workload by adrenoreceptor agonists for the estimation of potential antiischemic activity in a conscious rabbit model. *J Pharmacol Toxicol Methods* 1997; 37(3): 149-59.
7. Szekeres L, **Kovanecz I**, Papp JG. Delayed cardiac adaptation to stress moderates response to beta-adrenoceptor agonists. *J Mol Cell Cardiol* 1997; 29(5): A134.
8. **Kovanecz I**, Ábrahám A, Makay G, Lukács E, Szekeres L, Papp JGy. Delayed cardiac adaptation to ischaemic stress - limitation of infarct size in a rabbit model of ischaemia-reperfusion by a single dose of iloprost. 1997 . *J Mol Cell Cardiol* 1997; 29(5): A89.
9. Takase H, **Kovanecz I**, Mori T. et al. Acute and anti-ischemic actions of pranipidine in three animal models. *Asia Pacific Journal of Pharmacology* 2003; 16(1): 29-37.
10. Davila HH, Miranda-Sousa AJ, **Kovanecz I**, et al. Effect of bilateral cavernosal nerve resection on the histological alteration in the penile vascular system. *J Urol* 2005; 173(4S): 288.
11. Ferrini MG, **Kovanecz I**, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Effects of long-term vardenafil treatment on the development of fibrotic plaques in a rat model of Peyronie's disease. *BJU Int*. 2006 Mar; 97(3):625-33.
12. **Kovanecz I**, Ferrini MG, Vernet D, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Pioglitazone prevents corporal veno-occlusive dysfunction (CVOD) in a rat model of type 2 diabetes mellitus. *BJU Int*. 2006; 98:116-24
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15. Magee TR, **Kovanecz I**, Davila HH, Ferrini MG, Cantini L, Vernet D, Zuniga FI, Rajfer J, Gonzalez-Cadavid NF. Antisense and short hairpin RNA (shRNA) constructs targeting PIN (Protein Iinhibitor of NOS) ameliorate aging-related erectile dysfunction in the rat. *J Sex Med* 2007; 4(3):633-43.
16. **Kovanecz I**, Ferrini MG, Vernet D, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Aging-related copora veno-occlusive dysfunction in the rat is ameliorated by pioglitazone. *BJU Int* 2007; 100(4):867-74.
17. **Kovanecz I**, Rambhatla A, Ferrini MG, Rajfer J, Gonzalez-Cadavid NF. Long term sildenafil treatment ameliorates corpora veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats. *Int J Impot Res* 2007 Sep 20; [Epub ahead of print]
18. **Kovanecz I**, Rambhatla A, Ferrini MG, Rajfer J, Gonzalez-Cadavid NF. Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction that occurs after cavernosal nerve resection. *BJU Int.* 2008; 101(2):203-10.
19. Nolazco D, **Kovanecz I**, Vernet D, Ferrini MG, Gelfand B, Tsao J, Mage T, Rajfer J, Gonzalez-Cadavid NF. Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. *BJU Int* 2008 Feb 21; [Epub ahead of print]

**Non-experimental articles**

1. Rambhatla A, **Kovanecz I,** Ferrini M, Gonzalez-Cadavid NF, Rajfer J. Rationale For PDE5 Inhibitor Use Post Prostatectomy. *Int J Impot Res* 2008; 20(1):30-34. [Epub 2007 Aug 2.]

## D. Ongoing Research Support

None

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

| NAME | POSITION TITLE |
|---|---|
| Monica G. Ferrini | Assistant Professor |
| eRA COMMONS USER NAME (credential, e.g., agency login)<br>MFerrini306 | |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| University of Buenos Aires, Argentina<br>School Pharmacy and Biochemistry | M.Sc. | 1986 | Biochemistry |
| University of Buenos Aires, Argentina<br>School Pharmacy and Biochemistry | Ph.D. | 1995 | Physiology |

**A. POSITIONS**

1992-2001 Assistant Professor. University of Buenos Aires, Argentina
1994-2001 Member of the National Research Council of Argentina (CONICET). Current rank: Adjunct Investigator.
1999-2007 Research Associate, LA BioMed at Harbor UCLA, Los Angeles, CA
2004-on Assistant Research Professor, David Geffen School Med at UCLA, Department of Urology, Los Angeles, CA
2004-on Assistant Professor, Department Biomedical Sciences, College Health and Science, Charles R. Drew University, Los Angeles, CA.
2007-on Assistant Professor, Department of Internal Medicine, College of Medicine, Charles Drew University.

**HONORS**

1993. "Dr Juan Izquierdo Award" Argentine Society Experimental Pharmacology (SAFE)
1991-1995 "Scientific and Technological Awards University of Buenos Aires"
1996. 3rd place in the list of Young Oustanding Researcher, University of Buenos Aires. Argentina.
1997. "Mr Julio Lutfi Award" for the best young endocrinology researcher. Obtained diploma for second place . Argentine Society of Endocrinology and Metabolism.
2000. "AUA: Second place best poster category "Impotence"
2004. Travel Grant Award. Endocrine Society.

**B. PUBLICATIONS selected out of total of 52 papers**


1. **Ferrini M**, Magariños A.M, De Nicola AF (1990) Estrogens down-regulate type I but not type II adrenal corticoids receptors in rat anterior pituitary. J Steroid Biochem Mol Biol 35: 671-677.
2. **Ferrini M**, De Nicola AF. (1991) Estrogens up-regulate Type I and Type II glucocorticoid receptors in brain regions from ovariectomized rats. Life Sci 48 (26) 2593-2601.
3. **Ferrini M.**, González S, De Nicola AF. (1993) Estradiol increases glucocorticoid binding and glucocorticoid induction of ornithine decarboxilase in the rat spinal cord. Life Sci, 52: 677-685.
4. **Ferrini M.**, González S, Antakly T, De Nicola AF. (1993) Immunocytochemical localization of glucocorticoid receptors in the spinal cord: Effects of adrenalectomy, glucocorticoid treatment and spinal cord transection. Cell Mol Neurobiol, 13/4: 387-397.
5. **Ferrini, M.**, Lima A, De Nicola AF. (1995) Estradiol abolishes down regulation of glucocorticoid receptors in brain. Life Sci 57: 2403:2412.
6. **Ferrini, M.**, Grillo, C, Piroli, G, De Kloet, ER, De Nicola, AF. (1997) Sex difference in glucocorticoid regulation of vasopressin mRNA in the paraventricular hypothalamic nucleus. Cell Mol Neurobiol, 17: 671-686.
7. De Nicola, AF, **Ferrini, M.**, González, S, González Deniselle, MC, Grillo, C, Piroli, G, Saravia, S, De

Kloet, ER. (1998) Regulation of gene expression by corticoid hormones in the brain and spinal cord. J. Steroid Biochem Mol Biol, 65: 253-272.
8. **Ferrini, M**., Piroli G, Grillo,C, González-Deniselle,MC, Lima, A, Roig, P, De Nicola, AF. (1998) Effect of estrogen on the immunoreactivity of choline acetyl transferase (CHAT) and mRNA of GAP-43 in aged rats Act Physiol Latinoam 78 : 48 abt 23
9. **Ferrini, M.,** Piroli, G, Frontera M, Falbo, A, Lima, AE. (1999) Estrogen normalize the response to stress and increase glucocorticoid receptors immunoreactivity in aging rats. Neuroendocrinology 69 129-137
10. Gonzalez Deniselle, M.C., Lavista-Llanos, S., **Ferrini, M.,** Lima A.E., Roldan A.G., De Nicola A.F.: (1999) In vitro differences between astrocytes of control and wobbler mice spinal cord. Neurochem.Res**,** 24: 1531-1541
11. Bisagno, V., **Ferrini, M**., Rios H., Ziehr, L M and Wikinski S. I: (2000) Chronic corticosterone impairs inhibitory avoidance in rats: possible link with hippocampal CA3 dendritic atrophy. Pharmacol Biochem Behav. 66(2):235-40.
12. **Ferrini M.**, Magee, TR, Vernet D, Rajfer J, Gonzalez-Cadavid, NF (2001) Aging-related expression of inducible nitric oxide synthase and markers of tissue damage in the rat penis. Biol Reprod 64: 974-982.
13. **Ferrini M.**, Wang C, Swerdloff R, Vernet D, Sinha-Hikim A, Gonzalez-Cadavid NF. (2001) Aging related expression of inducible nitric oxide synthase (iNOS) and cytotoxicity markers in the rat hypothalamic regions associated with male reproductive dysfunctions. Neuroendocrinology 74: 1-11.
14. **Ferrini M.**, Vernet D, Magee TR, Shahed A, Qian A, Rajfer J, Gonzalez-Cadavid NF. (2002) Anfibrotic role of inducible nitric oxyde synthase. Nitric Oxide: Biol Chem 6:283-294
15. Magee TR, **Ferrini M**., Garban H, Vernet D, Mitani K, Rajfer J, Gonzalez-Cadavid NF (2002) Gene therapy of erectile dysfunction in the rat with penile neuronal nitric oxide synthase (PnNOS) cDNA. Biol Reprod. 67:20-28
16. **Ferrini M**., Bisagno V, Piroli G, Grillo, C, Gonzalez-Deniselle, MC, De Nicola AF. (2002) Effects of estrogens on choline-acetyltransferase immunoreactivity and GAP-43 mRNA in the forebrain of young and aging male rats. Cell Mol Neurobiol, 22:289-301.
17. Gonzalez-Cadavid NF, Magee TR**, Ferrini M**., Qian A, Vernet D, Rajfer J (2002) Gene expression in Peyronie's disease. In: "Update of Peyronie's disease", ed. by Nera A, Hellstrom W. Intn J Impot Res, 14(5): 361-374.
18. Vernet, D, **Ferrini MG,** Valente EG, Magee TR, Bou Gahrios G, Rajfer, J, Gonzalez-Cadavid, N.F. (2002) Effect of nitric oxide on the differentiation of fibroblasts into myofiobroblasts in the Peyronie's fibrotic plaque and in its rat model. Nitric oxide, Biol Chem 7:262-276.
19. **Ferrini M**, Magee TR, Vernet D, Rajfer J, Gonzalez-Cadavid NF (2002) Aging-related expression of inducible nitric oxide synthase and markers of tissue damage in the rat penis. Int J Impot Res 14:550 Editorial commentary
20. Magee, TR, **Ferrini, MG.**, Davila, H, Zeller, CB, Vernet, D, Sun, J, Lalani, R, Burnett, AL, Rajfer, J, Gonzalez-Cadavid, NF. (2003) The protein inhibitor of NOS (PIN) and the NMDAR receptor are expressed in the rat and mouse penile nerves and co-localize with penile neuronal nitric oxide synthase. Biol Reprod 68:478-88.
21. **Ferrini, MG.**, Magee, TR, Vernet, D, Rajfer, J, Gonzalez-Cadavid, NF. (2003) Penile neuronal nitric oxide synthase (PnNOS) and its regulatory proteins are present in hypothalamic regions involved in the control of penile erection. J Comp Neurol 458(1): 46-61.
22. Davila HH, **Ferrini MG**., Rajfer J, Gonzalez-Cadavid NF (2003) Fibrin as an inducer of fibrosis in the tunica albuginea of the rat: a new animal model of Peyronie's disease. Brit J Urol Int 91:830-838.
23. Valente EG, **Ferrini MG**, Vernet D, Qian A, Rajfer J, Gonzalez-Cadavid NF (2003) L-arginine and PDE inhibitors counteract fibrosis in the Peyronie's fibrotic plaque and related fibroblast cultures. Nitric Oxide, 9(4): 229-244.
24. **Ferrini M**, Davila HH, Valente EG, Gonzalez-Cadavid NF, Rajfer J. (2004) Aging-related induction of inducible nitric oxide synthase is vasculo-protective to the arterial media. Cardiovascular Res., 61(4):796-805.
25. Hikim AP, Vera Y, Vernet D, Castanares M, Diaz-Romero M, **Ferrini M**,Swerdloff RS, Gonzalez-Cadavid NF, Wang C (2005). Involvement of nitric oxide-mediated intrinsic pathway signaling in age-

related increase in germ cell apoptosis in male Brown-Norway rats. J Gerontol A Biol Sci Med Sci. 60(6):702-708.
26. **Ferrini MG**, Kovanecz I, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. (2006) Effects of long-term vardenafil treatment on the development of fibrotic plaques in a rat model of Peyronie's disease.BJU Int. 97(3):625-33.
27. **Ferrini MG**, Nolazco G, Vernet D, Gonzalez-Cadavid NF, Berman J. Increased vaginal oxidative stress, apoptosis, and inducible nitric oxide synthase in a diabetic rat model: implications for vaginal fibrosis. Fertil Steril. 2006 Oct;86 Suppl 4:1152-63.
28. De Nicola AF, Saravia FE, Beauquis J, Pietranera L, **Ferrini MG.** Estrogens and neuro-endocrine hypothalamic-pituitary-adrenal axis function.Front Horm Res. 2006;35:157-68.
29. **Ferrini MG,** Davila HH, Kovanecz I, Sanchez SP, Gonzalez-Cadavid NF, Rajfer J. Vardenafil prevents fibrosis and loss of corporal smooth muscle that occurs after bilateral cavernosal nerve resection in the rat.Urology. 2006 Aug;68(2):429-35
30. Kovanecz I, **Ferrini MG**, Vernet D, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Pioglitazone prevents corporal veno-occlusive dysfunction in a rat model of type 2 diabetes mellitus. BJU Int. 2006 Jul; 98(1):116-24.
31. Magee TR, Artaza JN, Ferrini MG, Vernet D, Zuniga FI, Cantini L, Reisz-Porszasz S, Rajfer J, Gonzalez-Cadavid NF. Myostatin short interfering hairpin RNA gene transfer increases skeletal muscle mass. J Gene Med. 2006 Sep;8(9):1171-81.
32. Paez Espinosa V, **Ferrini M**, Shen X, Lutfy K, Nillni EA, Friedman TC. Cellular co-localization and co-regulation between hypothalamic pro-TRH and prohormone convertases in hypothyroidism. Am J Physiol Endocrinol Metab. 2007 292(1):E175-86
33. **Ferrini MG,** Kovanecz I, Sanchez S, Vernet D, Davila HH, Rajfer J,Gonzalez-Cadavid NF. Long-Term Continuous Treatment with Sildenafil Ameliorates Aging-Related Erectile Dysfunction and the Underlying Corporal Fibrosis in the Rat. Biol Reprod. 2007, 73: 915-923
34. Magee TR, Kovanecz I, Davila HH, **Ferrini MG**, Cantini L, Vernet D, Zuniga FI, Rajfer J, Gonzalez-Cadavid NF. Antisense and short hairpin RNA (shRNA) constructs targeting PIN (Protein Inhibitor of NOS) ameliorate aging-related erectile dysfunction in the rat.J Sex Med. 2007 May;4(3):633-43.
35. Khorram O, Momeni M, **Ferrini M,** Desai M, Ross MG.In utero undernutrition in rats induces increased vascular smooth muscle content in the offspring. Am J Obstet Gynecol. 2007 May;196(5):486.e1-8.
36. Kovanecz I, **Ferrini MG**, Vernet D, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Ageing-related corpora veno-occlusive dysfunction in the rat is ameliorated by pioglitazone. BJU Int. 2007 Oct;100(4):867-74.
37. Rambhatla A, Kovanecz I, **Ferrini M**, Gonzalez-Cadavid NF, Rajfer J. Rationale for phosphodiesterase 5 inhibitor use post-radical prostatectomy: experimental and clinical review. Int J Impot Res. 2007 Aug 2; [Epub ahead of print]
38. Kovanecz I, Rambhatla A, **Ferrini M**, Vernet D, Sanchez S, Rajfer J, Gonzalez-Cadavid N. Long-term continuous sildenafil treatment ameliorates corporal veno-occlusion dysfunction (CVOD) induced by cavernosal nerve resection in rats. Int J Impot Res. 2007 Sep 20; [Epub ahead of print]
39. Kovanecz I, Rambhatla A, **Ferrini MG**, Vernet D, Sanchez S, Rajfer J, Gonzalez-Cadavid N. Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction that occurs after cavernosal nerve resection. BJU Int. 2007 Sep 20; [Epub ahead of print]
40. J.N. Artaza, R. Singh, **M.G. Ferrini,** M. Braga J. Tsao Myostatin promotes a fibrotic phenotypic switch in multipotent C3H 10T1/2 cells without affecting their differentiation into myofibroblasts. and N. Gonzalez-Cadavid. Journal of Endocrinology, 2008 Feb; 196 (2):235-249.
41. Cantini LP, **Ferrini MG**, Vernet D, Magee TR, Qian A., Gelfand RA, Rajfer J., Gonzalez-Cadavid NF: Pro-fibrotic role of myostatin in Peyronie's Disease J. Sex. Med. 2008 in press.
42. Nolazco G, Kovanecz I, Vernet D, **Ferrini MG**, Gelfand B, Tsao J, Magee T, Rajfer J, Gonzalez-Cadavid NF (2007) Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. BJU Int, in press

**C. OTHER SUPPORT**

Active

| | |
|---|---|
| Active/Pending: | Active |
| Project Number (Principal Investigator): | 1P20-MD-000545-01 (Sayed, Gary, Dean of COSH, Drew Univ.) |
| Source: | NIH |
| Title of Project (*and/or Subproject*): | Charles R Drew University College of Allied Health Undergraduate Program |
| Dates of Approved/Proposed Project: | 10/1/05 - 9/30/08 |

Annual Direct Costs / Percent Effort:

The College of Allied Health plans to enhance its current academic health and life science programs by creating an undergraduate program in Biomedical Science. To Achieve its goals, four areas will be implemented: 1. Faculty Development; 2. Physical Plant Development 3. Career Development. This would lead toward the eventual development of a strong educational biomedical research Bachelor degree program, that would serve as an educational pipeline to the College of Medicine at Drew or UCLA

| | |
|---|---|
| Active/Pending: | Active |
| Project Number (Principal Investigator): | Log #PR064756, PR#W91ZSQ-6289-N634 Pi: Gonzalez-Cadavid. |
| Source: | Department of Defense |
| Title of Project (*and/or Subproject*): | Modulation of stem cell differentiation and myostatin activity as an approach to counteract fibrosis in muscle dystrophy and regeneration after injury |
| Dates of Approved/Proposed Project: | 03/01/07-2/28/11 |
| Annual Direct Costs: | 197,247 |
| Person months (Calendar months): | 4.8 month |

Goals: The goal is to investigate in the mdx mouse a novel therapeutic approach for **DMD** based on the inhibition of myostatin **(Mst)** expression and/or activity, for the alleviation of fibrotic and fatty degeneration of the muscle, that would also facilitate the differentiation of transplanted dystrophin+ **(D+)** muscle-derived stem cells **(MDSC),** in order to ameliorate disease progression

Overlap: None.

| | |
|---|---|
| Active/Pending: | Pending Award |
| Project Number (Principal Investigator): | 1SC1GM038706-01. PI: Ferrini, Monica G |
| Source: | NIH |
| Title of Project (*and/or Subproject*): | Nitric oxide/cGMP modulation of corporal fibrosis caused by neuropraxia |
| Dates of Approved/Proposed Project: | 04/01/08-3/31/13 |
| Annual Direct Costs: | 200,000 |
| Person months (Calendar months): | 4.8 month |

Goals: To define whether PDE5 inhibitors alone or in combination with other drugs that also up-regulate the NO/cGMP pathway, correct not only the underlying histopathology of the corpora but also preserve the normal physiology of the tissue. In addition, to clarify the mechanism of these effects by determining a) to what extent different damaged tissues are affected by these agents; b) what role nitric oxide (NO) and cGMP have on correcting oxidative stress as a factor inducing corporal tissue damage after surgery; and c) what are the downstream targets of NO and cGMP when there is amelioration of corporal tissue damage.

Overlap: None.